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CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

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CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

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CLINICAL CHEMISTRY

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Seromucoid in Hepatobiliary Disease

Emanuel E. Mandel, Thomas L. Gorsuch, and Gerald E. Cooper

With the technical assistance of *Florence L. Jones*

AN INCREASE IN CIRCULATING carbohydrate-rich proteins has been known for some time to occur in diseases associated with neoplastic or inflammatory processes. These substances have been variously termed polypeptides (1–4), proteoses (5), mucoid-like material (6), seromucoid (7–10), and mucoprotein (11–15). It was only recently that the occurrence of a pathologic depression of the seromucoid (SM) level* was discovered, observed principally in hepatic disease (16–19). Thus, the incidence of a low SM level among 180 patients with either viral hepatitis or portal cirrhosis was about 80 per cent and compared favorably in diagnostic efficiency with the usual flocculation and other hepatic tests. On the other hand, only 2 per cent of 125 cases of obstructive jaundice showed a subnormal level, while 50 per cent had an increase (18, 19).

Since the test, in addition to its reputed diagnostic utility in such conditions as rheumatic fever and renal disease (20, 21), promised to be a useful means of distinguishing between "medical" and "surgical" jaundice (22)—an ever-present clinical problem—it was being considered by the authors for adoption in the routine hospital laboratory. As a necessary preliminary step, an attempt was made to reappraise the clinical significance of the test. The results of this study, which, in part, were in discord with the cited published findings, are reported here.

MATERIAL AND METHODS

Patients afflicted with various types of jaundice or hepatomegaly (other than that usually observed in congestive heart failure), or both,

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* This term is adopted in accordance with Meyer's recent classification (10).

were studied. Only those patients were included in this report whose diagnosis appeared established by autopsy, biopsy, surgical operation, or prolonged clinical observation. "Normal" controls were recruited from the hospital* and Public Health Service staffs; "hospital" controls included patients with such localized lesions as were unlikely to exert a significant systemic effect and to influence the SM level, such as healing fracture, cataract, or peptic ulcer.

Blood specimens for SM analysis were usually secured in the morning from subjects in the preprandial state, except for experiments designed to determine the effect of food intake on the SM concentration (see below). Serum was separated promptly upon adequate clot retraction and stored at $-10°$ to $-15°$, if not analyzed the same day. Results of the usual hepatic tests, including serum bilirubin (23), cephalin cholesterol flocculation (24), thymol turbidity (24), and alkaline phosphatase (25), were used for correlation with SM values only if the interval between respective blood collections was no greater than three days.

For the purpose of adequate comparison with Greenspan's data (18), his method of analysis—originally described by Simkin and coworkers (16)—was adopted, although some of its shortcomings were recognized early in the study (26). Serum (usually 4 ml., never less than 2 ml.) was treated with twice the volume of 0.6M perchloric acid. After exactly 10 minutes of standing and subsequent filtering through Whatman #5 paper, filtrate and 5% phosphotungstic acid (in 2N HCl) were, in a volume ratio of 3:1, placed in a 15 ml. centrifuge tube, gently mixed, and spun 15 minutes later at about 500 G. for 10 minutes. The seromucoid precipitate was washed once with the phosphotungstic acid solution and, after thorough drainage, dissolved in 3 ml. of 0.2N NaOH and 0.6 ml. of biuret reagent. After one hour's standing in a dark cabinet, the samples were read in a Beckman Model DU spectrophotometer at 540 mμ. Calculation of the SM content was made by reference to a standard curve obtained by means of solutions of 50 to 200 mg. of casein per 100 ml. of 0.2N NaOH, treated with biuret as indicated above.

RESULTS

Analysis of SM values determined among the normal controls revealed no striking difference between the fasting and nonfasting groups, if one discounts the low upper extreme in the nonfasting female group because of the small number of observations (Table 1). In individual cases, however, food intake did seem to influence the SM level, contrary to Green-

* Grady Memorial Hospital, Veterans Administration Hospital, and Emory University Hospital, Atlanta, Ga.

Table 1. Normal Range of Seromucoid

Authors	Number of observations	Sex	Food intake before analysis	Seromucoid level (mg./100 ml.)				
				Extreme values	Means	Standard deviation	Approximate 95% limits	Approximate 65% limits
Greenspan et al.	105	M	Not stated	38.9–78.7	59.2	7.3	48–75[a]	..
	80	F	Not stated	36.7–74.6	52.9	7.6	40–70[a]	..
Present authors	23	M	No	35.4–94.1	62.4	17.6	27–98	45–80[a]
	26	F	No	17.5–77.8	47.6	17.2	13–82	30–65[a]
	22	M	Yes	36.2–102.6	61.7	19.9	22–102	42–82
	11	F	Yes	27.6–55.5	38.8	9.2	20–57	30–48
	16[b]	M	No	31.2–98.8	70.3	20.3	30–111	50–91
	13[b]	F	No	23.4–93.3	51.9	18.7	15–89	33–71

[a] Range employed in diagnosis.
[b] Hospital controls.

span's experience (18), though not in a consistent manner (Fig. 1). Consequently, emphasis was placed on collecting blood for SM analysis from fasting subjects only. The range of values in the group of hospital controls was somewhat higher than among the "normals." In accordance with the findings of Greenspan and his associates (18), the range for females was distinctly lower than that for males in each control group. For each sex, however, the span of extreme values was considerably wider in this study than in the former authors' series, even though the latter included nearly four times as many observations. The lowest values in

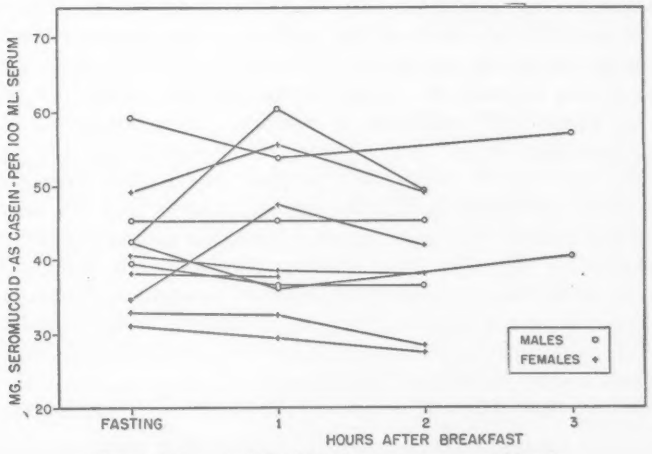

Fig. 1. Influence of food intake upon seromucoid level.

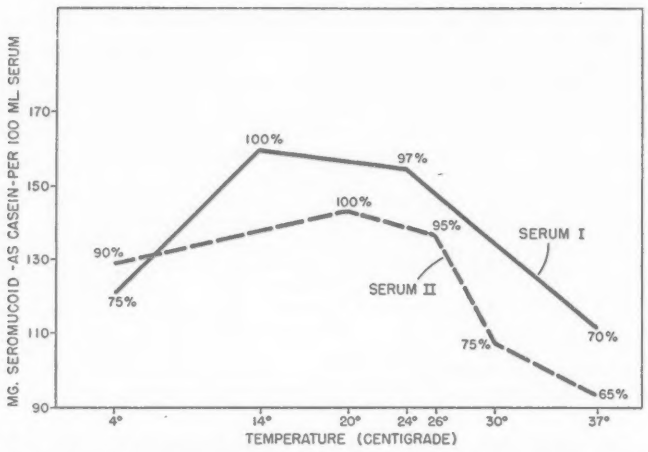

Fig. 2. Influence of temperature of perchloric-acid–serum mixture upon seromucoid yield.

the present control group were found in young and slender but obviously healthy nurses and medical technologists.

To a limited extent, these discrepancies can be explained by the difficulties encountered in analysis. The coefficient of variation (27) of duplicate determinations on 65 unselected consecutive sera was as high as 6.1 per cent.* In other words, the SM values that may be expected from a single serum range ±12% from the "ideal" or "true" value. If the latter were 50 mg./100 ml., 95% of the replicate determinations would fall between 44 and 56 mg. per 100 ml. The experimental error among values obtained from aliquots of a single serum analyzed several days apart was even greater, the coefficient of variation among aliquots of 10 such sera approaching 10 per cent. Among the factors (subsequently discovered) that may be responsible for this variation are differences in temperatures prevailing on different days of analysis (Fig. 2), as well as inadvertent changes in the procedure of pipetting and mixing serum and perchloric acid (26). The chief problem of precision is apparently inherent in this initial step in analysis, designed to separate the seromucoid from the other serum proteins. Analysis (in triplicates) upon 10 different perchloric acid filtrates yielded a coefficient of variation of 1%, and upon 30 different casein solutions, of 0.8% only.

In view of these limitations and the relatively small number of "nor-

* Winzler's method (11) yielded a coefficient of variation of 5 per cent.

mal" subjects studied, the 65 per cent confidence limits (27) were chosen to designate the normal range, which therefore constituted 45–80 mg./100 ml. for males and 30–65 mg./100 ml. for females. These extremes are in fairly close agreement with the 95 per cent confidence limits which represented the normal range of SM in Greenspan's series.

Among 44 cases of viral hepatitis (including 13 instances of presumed virus B infection), 17, or 39 per cent, showed a subnormal SM upon initial examination (Table 2). This percentage increased to 49 in the 29 cases in whom subsequent SM determinations could be secured. Nine of the above 44 patients were found to have complicating conditions (recent

Table 2. SEROMUCOID LEVELS IN 165 PATIENTS WITH HEPATOBILIARY DISEASE

Diagnosis	*No. of patients with:*		
	Increased values	*Normal values*	*Decreased values*
Acute hepatitis			
A or B virus	6(4)[a]	21(5)	17
Mononucleosis	1		
Leptospiral	1	1	
Amoebic	1		
Septic	1		
Toxic		1	
Chronic liver disease			
Fatty metamorphosis	1(1)	1	
Alcoholic cirrhosis	18(16)	17(11)	6(1)
Postnecrotic cirrhosis			3
Cardiac cirrhosis		1	1
Hemochromatosis	1		
Sarcoidosis	2		1
Biliary obstruction			
Benign			
Acute cholecystitis	4		
Chronic cholecystopathy	7		
Common duct stone	6	2	
Common duct stricture	1	1	
Congenital duct stenosis	1	1	
Malignant			
Carcinoma[b]	22	4	
Hepatoma	2	2	
Lymphoma	2		
Hemolytic jaundice			
Congenital spherocytosis	3(2)		
Sickle cell anemia	2(1)	1	
Transfusion reaction	1		
Malaria		1	

[a] Parentheses denote complicating illness (in hepatitis and hemolytic jaundice groups).

[b] Carcinoma of gastrointestinal tract (4 cases), pancreas (12), biliary tract (2), kidney (1), and of unknown origin (7), causing jaundice and/or metastatic hepatomegaly.

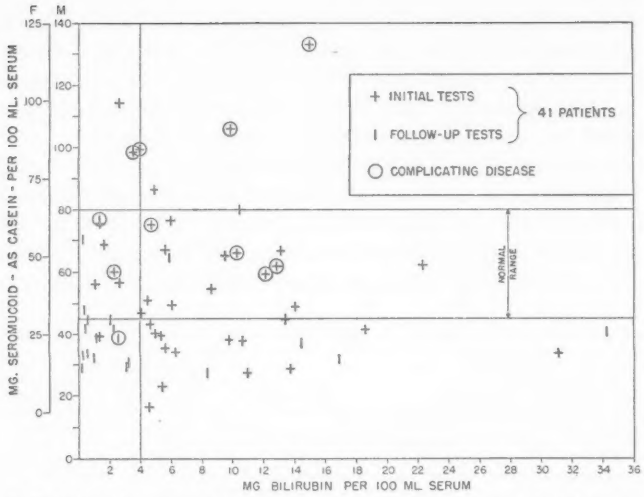

Fig. 3. Relation of seromucoid to severity of viral hepatitis as denoted by serum bilirubin. In all but 9 patients, the serum bilirubin was above 4 mg./100 ml. on initial examination. Most of the corresponding seromucoid values were either normal or low. χ^2 test (27) failed to suggest correlation between seromucoid and bilirubin.

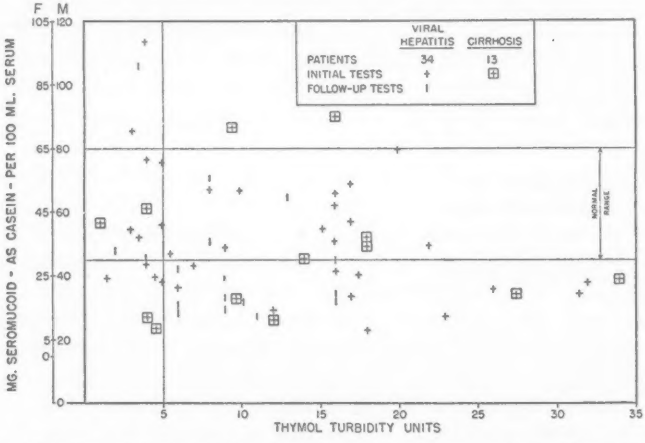

Fig. 4. Relation of seromucoid to thymol turbidity in uncomplicated hepatic disease. Less than one third of the cases showed, upon initial testing, a normal thymol turbidity (below 5 units) while more than one half had a normal or elevated (negative) seromucoid. In 6 instances, seromucoid was positive and thymol negative; in 9, both tests failed to indicate hepatic disease. χ^2 test (27) failed to suggest correlation between seromucoid and thymol turbidity.

surgical operations, pneumonia, pyelonephritis, mastitis, pregnancy), to which the failure of SM depression could be ascribed. Even if these cases were eliminated, reducing the group to 35 cases, there was still about one half of these which yielded a normal or, in 2 instances, elevated SM without evidence of an extrahepatic "SM-raising" process. While some of these patients had suffered a mild attack or were already in the recovery stage, as indicated by the low serum bilirubin level (Fig. 3), about one half of them showed considerable jaundice, malaise, and associated laboratory evidence of hepatic damage (Fig. 4) at the time of the SM test.

Absence of SM depression appeared to characterize a group of 6 patients presenting an acute hepatitis syndrome secondary to certain systemic disorders: infectious mononucleosis, leptospirosis (two), amoebiasis, acute bacterial endocarditis, and intoxication with bootleg whiskey.

Since complications likely to augment the SM level (chronic pyelonephritis, pneumonia, subphrenic abscess, lymphopathia venereum, urticaria, and delirium tremens) occurred in the majority of the 41 patients with alcoholic cirrhosis, it was not surprising that only 6 (15 per cent) showed a subnormal SM level. In 2 instances, a high initial SM was associated with prolonged fever and leukocytosis, attributed to enterogenous bacteremia; subsidence of these signs of inflammation weeks later was accompanied by SM depression. Of the 14 patients in this group who showed no evidence of complications, there were still 2 with an increased and only 6 (43 per cent) with a low SM. The latter finding was also encountered in the 3 cases of postnecrotic cirrhosis, in 1 of the 2 cases of cardiac cirrhosis, and in 1 of 3 cases of sarcoidosis. SM was elevated in a patient with hemochromatosis but apparently not affected by fatty infiltration of the liver.

Thus, the incidence of significantly reduced SM levels in the group of

Table 3. EFFICIENCY OF SEROMUCOID AND OF THREE OTHER SERUM TESTS IN THE DIAGNOSIS OF HEPATOBILIARY DISEASE

Diagnosis	*Seromucoid*			*Cephalin flocculation*			*Thymol turbidity*			*Alkaline phosphatase*		
	Above normal (%)		*Below normal (%)*	*Above 2+ (%)*		*0–2+ (%)*	*Above 5 units (%)*		*Below 5 units (%)*	*4–10 units (%)*		*Above 10 units (%)*
Viral hepatitis	14	(44)[a]	39	82	(39)		77	(39)		27	(22)	5
Cirrhosis	40	(47)	21	62	(39)		63	(38)		32	(19)	16
Benign obstruction	83	(23)	0		(20)	80		(22)	82	43	(14)	43
Malignant obstruction	81	(32)	0		(24)	79		(26)	81	55	(22)	27

[a] Parentheses denote number of patients in whom test was performed.

"primary" hepatic diseases was only 28 per cent, far below expectations based on Greenspan's reports (17, 18). This disappointment was enhanced upon comparison with some of the usual hepatic tests, performed concomitantly with the SM test. The cephalin flocculation and thymol turbidity tests were positive approximately twice as often in viral hepatitis and nearly three times as often in cirrhosis (Table 3). Even after exclusion of cases with extrahepatic complications, the incidence of diagnostic SM levels was still below 50 per cent. These findings are strikingly different from Greenspan's, who reported the incidence of a subnormal SM in hepatitis and cirrhosis as being 88 per cent and 70 per cent, respectively, while the cephalin flocculation and thymol turbidity tests gave less frequent positive results: 48 and 68 per cent, respectively, in hepatitis, and 41 and 39 per cent in cirrhosis.

With regard to the serum alkaline phosphatase, experiences of the authors correspond more closely with those of Greenspan's in that this test yielded a positive result less often than the SM test (results of the two series are not directly comparable because of the differences in critical enzyme levels used).

On the other hand, in 55 cases of biliary obstruction (Table 2; Fig. 3) not a single instance of a subnormal SM was encountered while the two routine flocculation tests gave 20 per cent of "false" positive reactions (Table 3); the alkaline phosphatase failed in 27 to 43 per cent of the cases to show a marked elevation. In nearly three times as many cases of obstructive jaundice, Greenspan and his associates found the incidence of a subnormal SM level to be less than 3 per cent (19).

Hence, a subnormal SM level in the presence of jaundice is almost specific for a primary parenchymal disease, a statement that cannot be made for any of the commonly used hepatic tests. The SM test may therefore be most valuable in problems involving the differentiation of "medical" from "surgical" jaundice when the usual clinical and laboratory technics give equivocal results: a subnormal level weighs heavily in favor of "medical" jaundice. The utility of the test was clearly enhanced by the use of follow-up determinations (17, 21); not infrequently, when the first test gave a normal or borderline SM value, examination a few days later revealed a distinct tendency of the SM level to rise or fall, suggesting obstructive or parenchymal disease, respectively. A single normal or increased SM concentration per se was of little help in ruling out parenchymal disease, even in the absence of extrahepatic complication. However, when such a diagnosis appeared to be well established, the finding of an elevated SM suggested the possibility—and, in several cases, initiated the discovery—of either an extrahepatic (inflammatory

or neoplastic) complicating illness or an icterogenic agent other than the hepatitis virus (mononucleosis, leptospirosis, etc.). With about 80 per cent of both benign and malignant forms of biliary obstruction yielding high SM levels, the test could not be utilized to render a preoperative differentiation of the nature of the obstructive lesion.

DISCUSSION

Analysis of the data presented permits one to attribute a definite diagnostic value only to the finding of a subnormal SM level. Such a finding, in association with jaundice or hepatomegaly, or both, and in the absence of a nephrotic syndrome (20), of excessive hyperglobulinemia, and, perhaps, of certain endocrinopathies (19), is almost pathognomonic of hepatocellular disease. Normal or elevated levels are of very limited value and only occasionally make a decisive diagnostic contribution. In particular, high levels do not spell neoplastic disease, an observation which has been made in the past by Henry and coworkers (13) and, most recently, by Rhees and his associates (15). Although these investigators disregarded the existing sex difference in the normal SM concentration (Table 1) (17), their conclusions conform to Greenspan's (19) and the authors' experiences.

Recognition of abnormal (high or low) levels is obviously dependent

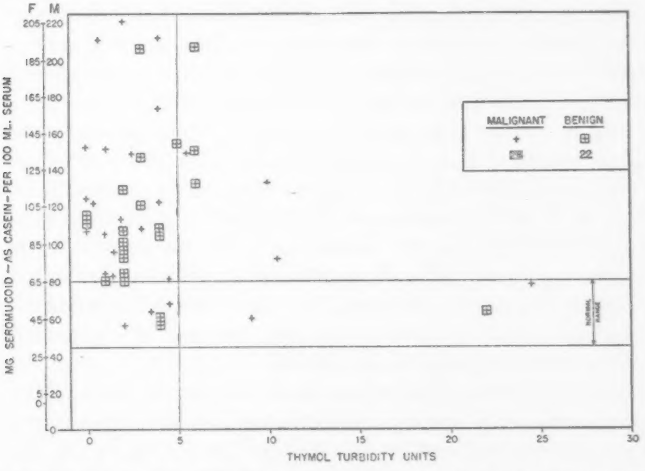

Fig. 5. Relation of seromucoid to thymol turbidity in biliary obstruction. The seromucoid test gave no false-positive (subnormal) result but the thymol turbidity was increased in 9 cases.

upon the definition of the "normal range," which in the present study was quite arbitrarily established as the 65 per cent confidence limits of all normal values encountered. Had the 95 per cent limits been used (Table 1), which would be more acceptable from a statistical point of view, the incidence of subnormal values would have been far lower than above recorded, whereas the number of high levels would have decreased but slightly (Figs. 3–5). The absence of any apparent false-positive results furnished by the values herein designated as subnormal lends some justification to the choice of the normal range as presented; additional support stems from the close similarity to the normal extremes employed by Greenspan and coworkers (17).

However, these, as well as other problems of a more technical nature mentioned initially, throw a suspicious light on the reliability of the analytic method and on the biologic significance of the results. Seromucoid obviously does not represent a single substance but rather a serum fraction whose separation from the other serum proteins by means of perchloric acid or other reagents (sulfosalicylic acid, trichloracetic acid [5, 11]) is quite arbitrary. This fact is evident from the marked changes in SM values that can be produced by relatively small alterations in the concentration of either acid or serum proteins or in the proportion of acid to serum (11, 26, 28). Increases in serum globulin such as are observed in sarcoidosis or multiple myeloma apparently tend to reduce the SM level (18), presumably through physicochemical action.

Electrophoretic analysis of perchloric-acid–serum filtrates revealed the presence of at least 3 peaks (MP-1, MP-2, and MP-3) (11, 29). Chemically, carbohydrate (galactose-mannose), hexosamine and protein (measured as tyrosine) constituted, aside from lipids, the chief components, in a ratio of 15.1:11.9:4.2 (by weight, on a moisture-free basis). Since the carbohydrate:protein (tyrosine) ratio was apparently maintained when the SM (tyrosine) level was markedly elevated in patients with cancer or pneumonia, it was concluded that the material responsible for the increase was chemically similar (but not necessarily identical!) to that isolated from normal serum (12). However, subnormal SM values (expressed as casein) encountered in liver disease were associated with increased carbohydrate:protein ratios (17).

With the use of ammonium sulfate fractionation, an electrophoretically and ultracentrifugally homogeneous plasma mucoid (M-1, corresponding to MP-1) was prepared by Winzler and his associates (30, 31). This had a molecular weight of 44,100, the mobility of α_1-globulin, and an isoelectric point of *p*H 1.8 at a buffer *p*H of 8.6; it contained approximately 26 per cent of carbohydrate (including hexosamine) and 10 per

cent of "sialic acid" (32). The substance was finally isolated in crystallized form by Schmid from Cohn's fraction VI (33). Variations of this "acid glycoprotein" due to disease have, in the absence of a practical clinical method, not been reported.

It is thus obvious that seromucoid, as determined in perchloric acid filtrates, does not represent a chemical or biologic entity. There is much question as to whether increases or decreases of certain of its constituents —protein, hexosamine, hexose polysaccharide, or others—necessarily reflect equivalent fluctuations of the whole complex. Consequently, any speculation about the mechanism responsible for changes in SM concentration found in various disease states are bound to be superficial and fraught with error.

It has been suggested that serum glycoproteins originate from the depolymerization of ground substance of connective tissue (34, 35). This process, normally under enzymatic and hormonal control, may be augmented by certain alterations in tissue metabolism, particularly those associated with destruction or proliferation, causing an excess of soluble, lower-molecular substances to diffuse into the circulation (34–38) and be excreted in the urine (39). These substances are only in part represented by seromucoid which is devoid of hexuronic acids (10, 11, 30, 32, 33), the regular constituents of tissue glycoprotein (40). Hexuronic acids (41, 42), as well as polysaccharides other than those contained in seromucoid, are normally present in serum (37) and may be pathologically increased. The decrease in the concentration of seromucoid (but not of those carbohydrate derivatives) occurring in liver disease suggests that the liver may act either as another source of SM or as an organ capable of removing it from the blood stream. The former possibility, implying inadequate synthesis by the diseased liver (18), is suggested by Werner's observations on the serum hexosamine response of rabbits subjected to blood loss; the usual increase failed to occur when liver damage was produced by means of phosphorus or chlorobenzene (43). On the other hand, total protein-bound hexose polysaccharides have been found in normal or increased serum concentrations in viral hepatitis (17). The role of the liver as a source of SM is also suggested by the finding of reduced α_1-globulin in viral hepatitis (44, 45) and by the observation, based on C^{14} isotope studies, that most of the major serum protein fractions stem from the liver, except for γ-globulin and minute amounts of β- and α-globulins (46, 47).

As a working hypothesis, connective tissue and the hepatic parenchyma may be regarded as two independent sources of seromucoid, each source having a different type of response to physiologic stimulation or injury:

the former chiefly in the form of an increase, the latter by way of a decrease. The SM level, as analyzed at any given time, constitutes the sum of these responses. Thus, only marked preponderance of the response of one source over that of the other will result in a distinctly abnormal and diagnostically useful level. The combination of two severe responses, i.e., pneumonia and viral hepatitis, is liable to yield a normal level.

This theory leaves open the question of endocrine factors influencing the SM level (48–51) and, indeed, the distinct possibility that there may be many different sites of origin of SM and of different glycoproteins and their individual constituents, with different degrees or types of response to certain pathologic processes. Further clinical or physiologic studies of seromucoid would seem to be of little avail without the attainment of far greater precision in chemical identification and in analytic measurement of the substances involved. Any attempt at classification of diseases by seromucoid levels (19) must therefore be regarded as preliminary and subject to future revision.

SUMMARY AND CONCLUSIONS

Seromucoid was determined in 165 patients with jaundice or hepatomegaly, or both. Subnormal seromucoid concentrations were found in 39 per cent of 44 cases of viral hepatitis and in 15 per cent of 41 cases of alcoholic cirrhosis. This incidence increased to 50 and 43 per cent, respectively, when patients with complicating extrahepatic disease processes were excluded. No subnormal values were encountered in 55 cases of benign and malignant biliary obstruction which, however, yielded about 80 per cent of elevated values.

The empirical data point to the lower limit of the normal seromucoid range as providing a useful dividing line between obstructive and parenchymal jaundice: the finding of a subnormal level is strongly indicative of the latter. Normal or elevated levels may only occasionally be expected to make a decisive diagnostic contribution.

A more definitive evaluation of the clinical utility of this type of test must await improvements in analytic standardization, based on a more detailed knowledge of the chemical and physiologic identity of the compound than is available at present.

REFERENCES

1. Hahn, A., *Biochem. Z.* **121**, 262 (1921).
2. Wolff, E., *Ann. méd.* **10**, 185 (1909).
3. Cristol, P., and Puech, A., *Bull mém soc. méd. hôp. Paris* **50**, 1826 (1926).
4. Goiffen, R., and Spaey, J., *Bull. Soc. chim. biol.* **16**, 1675 (1934).

5. Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.* **4**, 417 (1944).
6. Mayer, K., *Z. physiol. Chem.* **275**, 16 (1942).
7. Zannetti, C. U., *Gazz. chim. ital.* **33**, 160 (1903).
8. Bywaters, H. W., *Biochem. Z.* **15**, 322 (1909).
9. Rimington, C., *Biochem. J.* **34**, 931 (1940).
10. Meyer, K., Rutgers Univ. Press, New Brunswick (1953).
11. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.* **27**, 609 (1948).
12. Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.* **27**, 617 (1948).
13. Henry, R. J., Berkman, S., Little, M. S., and Winzler, R. J., *J.A.M.A.* **147**, 37 (1951).
14. Winzler, R. J., *Advances in Cancer Research* **1**, 503 (1953).
15. Rhees, M. C., Ellerbrook, L. D., and Lippincott, S. W., *Am. J. Clin. Pathol.* **24**, 774 (1954).
16. Simkin, B., Bergman, H. C., and Prinzmetal, M., *Am. J. Med.* **6**, 734 (1949).
17. Greenspan, E. M., Tepper, B., Terry, L. L., and Schoenbach, E. B., *J. Lab. Clin. Med.* **39**, 44 (1952).
18. Greenspan, E. M., and Dreiling, D., *A.M.A. Arch. Internal Med.* **91**, 474 (1953).
19. Greenspan, E. M., *A.M.A. Arch. Internal Med.* **93**, 863 (1954).
20. Kelley, V. C., Good, R. A., Glick, D., and Ochs, M. J., *J. Clin. Invest.* **29**, 1500 (1950).
21. Kelley, V. C., Adams, F. H., and Good, A., *Pediatrics* **12**, 607 (1953).
22. Popper, M., and Steigmann, F., *Ann. Internal Med.* **29**, 496 (1948).
23. Malloy, M. T., and Evelyn, K. A., *J. Biol. Chem.* **119**, 491 (1937).
24. Neefe, J. R., Gambescia, J. M., Gardner, H. T., and Knowlton, M., *Am. J. Med.* **24**, 600 (1950).
25. Bodansky, A., and Jaffe, M. L., *Proc. Soc. Exp. Biol. Med.* **31**, 107 (1933).
26. Mandel, E. E., Jones, F. L., and Gorsuch, T. L., *Federation Proc.* **13**, 258 (1954).
27. Mainland, D., *Elementary Medical Statistics*, Philadelphia, Saunders, 1952.
28. Weimer, H. E., and Moshin, J. R., *Am. Rev. Tuberc.* **68**, 594 (1953).
29. Mehl, J. W., Humphrey, J., and Winzler, R. J., *Proc. Soc. Exp. Biol. Med.* **72**, 106 (1949).
30. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.* **185**, 561 (1950).
31. Smith, E. L., Brown, D. M., Weimer, H. E., and Winzler, R. J., *J. Biol. Chem.* **185**, 569 (1950).
32. Odin, L., and Werner, I., *Acta Soc. Med. Upsaliensis* **57**, 227 (1952).
33. Schmid, K., *J. Am. Chem. Soc.* **75**, 60 (1953).
34. Gersh, I., and Catchpole, H. R., *Am. J. Anat.* **85**, 457 (1949).
35. Catchpole, H. R., *Proc. Soc. Exp. Biol. Med.* **75**, 221 (1950).
36. Engel, M. B., *Arch. Path.* **53**, 339 (1952).
37. Shetlar, M. R., Payne, R. W., Bullock, J. A., Patrick, D. R., Hellbaum, A. A., and Ishmael, W. K., *J. Clin. Invest.* **32**, 1208 (1953).
38. Maddock, C. L., *Proc. Soc. Exp. Biol. Med.* **85**, 598 (1954).
39. Engel, M. B., and Catchpole, H. R., *Proc. Soc. Exp. Biol. Med.* **84**, 336 (1953).
40. Cantarow, A., and Schepartz, B., *Biochemistry*, Philadelphia, Saunders, 1954.
41. Stary, Z., and Yuvanidis, M., *Biochem. Z.* **324**, 206 (1953).
42. Saltzman, A., Caraway, W. T., and Beck, I. A., Metabolism, *Clin. and Exp.* **3**, 11 (1954).
43. Werner, I., in *Acta Physiol. Scand.* **19**, 27 (1949) (abstr.).
44. Brante, G., *Scand. J. Clin. & Lab. Invest.* **4**, 293 (1952).
45. Mandel, E. E., and Cooper, G. R., Unpublished observations.
46. Miller, L. L., and Bale, W. F., *J. Exp. Med.* **99**, 125 (1954).
47. Miller, L. L., Bly, C. G., and Bale, W. F., *J. Exp. Med.* **99**, 133 (1954).
48. Boas, N. F., and Soffer, L. J., *J. Clin. Endocrinol.* **11**, 39 (1951).
49. Mancini, J. C., and Garberi, J. C., *J. Clin. Endocrinol.* **12**, 965 (1952).
50. Kelley, V. C., Kirschvink, J. F., and Ely, R. S., *Am. J. Physiol.* **171**, 738 (1952).
51. Weimer, H. E., and Redlich-Moshin, J., *Proc. Soc. Exp. Biol. Med.* **84**, 34 (1953).

Estimation of Hydrocortisone Secretion

Method of Calculation from Urinary-Excretion Data

Robert H. Silber

In 1938, Anderson, Haymaker, and Joseph (1) reported the finding of increased concentrations of cortin in the blood and urine of patients with Cushing's syndrome. Since then, as methods for the determination of cortical steroids have been improved, they have been applied to the urine and plasma of patients as a measure of adrenocortical activity. The reaction of 17,21-dihydroxy-20-ketosteroids with phenylhydrazine (2) has been applied to the analysis of plasma (3) and of urine (4) in Addison's disease and in Cushing's syndrome. In general, hyperactivity or hypoactivity of the adrenal cortex is reflected by increased or decreased plasma concentrations and excretion of 17,21-dihydroxy-20-ketosteroids. However, there may be conditions in which the output of the adrenal cortex is altered without demonstrable changes in the corticosteroid content of blood or urine. Therefore, it seemed desirable to devise a procedure which could be used clinically to estimate the adrenal output of hydrocortisone.

THEORETICAL CONSIDERATIONS

If a mathematical relationship between total (endogenous plus exogenous) hydrocortisone in the body and the fraction found in the urine can be established after administration of graded doses of hydrocortisone, it should be possible to extrapolate back to zero exogenous hydrocortisone and thereby determine the fraction of the endogenous hydrocortisone that is excreted. The calculation of endogenous production would then follow directly, after measurement of the basal steroid excretion in a given period of time. In theory, to accomplish this, the exogenous hydro-

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cortisone should be administered slowly into the adrenal vein to mix intimately with the endogenous steroid and to share its metabolic fate in the body. This mode of administration is obviously impractical and due to the required manipulation of the subject would not necessarily reflect the normal output of the adrenal cortex. Oral administration has been found to yield surprisingly satisfactory results, with a constant fraction of the exogenous steroid detected in the urine by the procedure of Silber and Porter (5) over the dosage range studied.

MATERIAL AND METHOD

Since, by the analytic procedure employed, we find that the increase in excretion of 17,21-dihydroxy-20-ketosteroids after oral administration of hydrocortisone is essentially complete within 8 hours, this period appeared to be satisfactory for the experimental demonstration of the method. In practice a 24-hour period has also been used and may be preferred.

On 4 consecutive days 5 male laboratory workers consumed a light breakfast at 7:00 A.M., voided at 8:30 A.M., ingested 0, 10, 25 or 50 mg. of hydrocortisone (free alcohol) with about 200 ml. of water, and collected all urine until 4:30 P.M. The subjects were permitted to have lunch at 11:30 A.M. Creatinine determinations were performed on all urines to check the adequacy of the collections. Phenol was added as preservative and samples of urine were frozen until all could be treated with glucuronidase[1] and assayed simultaneously.

Effects of Drugs

One subject (No. 2) ingested additional doses of 75 and 100 mg. (Table 1). This subject was studied again several weeks later and in subsequent weeks was used to determine the effect of several drugs on adrenocortical activity. During one series of collections, 2.7 Gm. of aspirin were ingested daily in 3 equal portions at 0 time, 2½ hours, and 5 hours; in a second, 300 mg. of ascorbic acid were taken, again in 3 equal portions; in a third, 100 mg. of Pyribenzamine, in 2 equal portions, were ingested; and in a fourth, 40 units of ACTH (Armour H. P. ACTHAR Gel) was administered daily by intramuscular injection (Table 2).

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[1] Due to possible changes in the enzyme preparation, we now check each lot before use and usually find that 5–10 times as much enzyme as indicated in the published method (5) is needed.

RESULTS

Four of the subjects (see Table 1) excreted 24–32 per cent of the oral hydrocortisone as 17,21-dihydroxy-20-ketosteroids. The fifth subject excreted only 13.6 per cent. By applying these percentages to the 8-hour basal excretion values the secretion of hydrocortisone by the adrenals of these subjects in 8 hours may be estimated in milligrams. However, it may be more desirable to employ an extrapolated basal value for this calculation because analytic errors are minimized after administration of the steroid. In most instances the extrapolated value and the actual basal excretion were not significantly different.

The adrenal output of these subjects averaged 13.8 mg. of hydrocortisone in the 8-hour period. When the above approach was applied to a group of 20 males (21–35 years of age) studied with Dr. Bacon Chow

Table 1. URINARY EXCRETION OF 17,21-DIHYDROXY-20-KETOSTEROIDS BY HUMAN SUBJECTS
Milligrams Free plus Conjugated in 8 Hours

Hydrocortisone dose (mg.)	*Subject 1*	*Subject 2*[a]	*Subject 3*	*Subject 4*	*Subject 5*
0	5.3 (4.7)	3.8 (2.7)	2.9 (2.8)	3.0	2.95
10	7.9	5.73	5.15	5.35	4.2
25	12.6	10.3	9.3	9.0	6.2
50	20.6	18.0	15.9	15.4	10.6
% excreted	31.8	30.4	25.2	24.0	13.6
Adrenal output (mg./8 hr.)	14.8	8.9	11.1	12.5	21.7

Parenthetical figures are extrapolated basal excretion values which were used in the calculations.

[a] After 75-mg. dose, 25.6 mg. excreted; after 100 mg. dose, 32.1 mg.

Table 2. EFFECT OF SEVERAL DRUGS ON ADRENAL OUTPUT OF SUBJECT 2
Milligrams free plus conjugated in 8 hours

Hydrocortisone dose (mg.)	*Control (first)*	*Aspirin (2.7 Gm.)*	*Ascorbic acid (300 mg.)*	*Pyribenzamine (100 mg.)*	*ACTH (40 units)*	*Control (second)*
0	2.94 (2.9)	4.0	4.15 (4.0)	4.2	11.5	3.65
10	5.6	6.63	6.4	7.07	16.9	5.7
20	8.2	9.25	9.3	9.2	22.6	9.05
30	11.2	..	11.9	12.7	..	11.05
% excreted	27.1	26.3	25.6	27.3	54.8	24.1
Adrenal output (mg./8 hr.)	10.7	15.2	15.6	15.4	21.0	15.1

Parenthetical figures are extrapolated basal excretion values which were used in the calculations.

of Johns Hopkins (6), 24-hour urine collections were made and the adrenal output was calculated to be 21.9 ± 8.2 mg. (s.d.) per 24 hours. In this study (6) only 2 urine collections were made—one before and one after administration of 40 mg. of hydrocortisone.

Calculations

For purposes of illustration, the data obtained on subject No. 2 (see Table 2) during the first control period are shown below:

$$\frac{\text{mg. excreted after test dose} - \text{basal excretion in mg.}}{\text{test dose in mg.}} = \text{fraction excreted}$$

$$\frac{5.6 - 2.9}{10} = .27$$

$$\frac{8.2 - 2.9}{20} = .265$$

$$\frac{11.2 - 2.9}{30} = .277$$

$$\text{Av.} = \overline{.271} \text{ of dose excreted}$$

$$\text{Then, } \frac{\text{basal excretion in mg.}}{\text{fraction excreted}} = \text{adrenal output (mg. in 8 hours)}$$

$$\frac{2.9}{0.271} = 10.7 \text{ mg.}$$

It is also possible to determine the fraction excreted by the application of simultaneous equations. (In dogs we find this procedure necessary.)

a = exogenous hydrocortisone, mg. (known)
b = a constant
x = fraction excreted
y = steroid excreted
$(a + b)x = y$
$(10 + b)x = 5.6$
$(20 + b)x = 8.2$

Then, subtracting the first from the second,

$$10x = 2.6$$
$$x = 0.26 \text{ or } 26\%$$

This fraction is then applied to the analytically determined basal excretion value (as in dogs), or, if desired, to the extrapolated basal value (as in man).

DISCUSSION

The quantity of hydrocortisone secreted by the adrenal glands of human subjects has been the subject of a great deal of speculation, but relatively little pertinent data have been published. Weichselbaum (7) has found that administration of approximately 40 mg. of hydrocortisone to adrenalectomized subjects in a 24-hour period brings their plasma concentrations into the normal range. This value compares favorably with the average values reported in this paper, 13.8 mg. in the 8-hour period, or 21.9 mg. in 24 hours (6).

Comparative Evaluation of Procedures

In the dog the urinary analyses are less accurate, due in part to the low excretion of 17,21-dihydroxy-20-ketosteroids (basal about 0.2–0.3 mg. per day) but application of the procedure herein described indicates that the adrenals of a 10–12-Kg. dog secrete about 1–2 mg. per day, and after 80 units of ACTHAR daily intramuscularly this increases to 5–6 mg. Since it has been found in our laboratory that the 24-hour excretion of 17-hydroxycorticoids by adrenalectomized dogs was increased from zero to the normal range of 0.2–0.3 mg. after administration of 1 or 2 mg. of hydrocortisone, it appears that the procedure is valid and can yield satisfactory results in the dog. Analysis of blood from the adrenal vein of intact 14–20-Kg. dogs by Hume and Nelson (8) revealed an adrenal output of 0.6–2.0 μg. per minute. A dog secreting a total of 2 mg. in a 24-hour period would have an average secretion of 1.4 μg. per minute from the 2 adrenals, so the results of the direct and indirect procedures are in reasonably satisfactory agreement.

Pharmacologic Influences

The data on the effect of aspirin, ascorbic acid, and Pyribenzamine are presented here more to illustrate the method than to determine whether these materials influence adrenal output, but it does appear that these substances did not significantly influence adrenal activity. ACTH, as expected, increased adrenal output to 21 mg. in 8 hours (and 30 mg. in 12 hours). The shift of the fraction of steroid excreted from 25 or 30 per cent to 55 per cent during ACTH treatment is very interesting and suggests that ACTH may not only stimulate secretion by the adrenal cortex but may also influence hydrocortisone metabolism. When sufficient data are obtained on patients it may be found that the fraction excreted in the urine has diagnostic significance.

Absorption and Metabolism

Oral administration of the steroid appears to be justified. The normal urinary excretion of 17,21-dihydroxy-20-ketosteroids in man as measured here is about 6.6 mg. per 24 hours, with only about 5 per cent in chloroform-soluble form—that is, not conjugated (5). After oral administration of hydrocortisone the proportion of free to conjugated steroid is relatively unchanged. Furthermore, after oral or intravenous administration of 25 or 100 mg. to dogs (9), the excretion of total (free plus conjugated) 17,21-dihydroxy-20-ketosteroids is essentially the same. Peterson (10) has reported similar findings in men given hydrocortisone orally or intravenously. Therefore, it is reasonable to conclude that not only is the hydrocortisone practically quantitatively absorbed from the intestinal tract but also its metabolism is similar to that of the endogenous steroid.

CONCLUSIONS

Although one may have reservations regarding the absolute validity of results obtained with the procedure described, it nevertheless appears adequate to reveal changes in adrenal output.

The experimental approach used in this study may be applied to the study of other substances, provided certain conditions are fulfilled. Thus, if one can exclude the substance in question from the diet for several days, if an excretion product can be accurately determined in the urine, if the subject is in a reasonably steady state, and if the substance can be administered in graded doses, it may be possible to estimate the formation or synthesis of that substance in the body.

SUMMARY

A method has been described for the estimation of the secretion of hydrocortisone by the adrenals of man, based exclusively upon urinary-excretion data. In an 8-hour period (8:30 A.M.–4:30 P.M.) 5 subjects secreted an average of 13.8 mg. of hydrocortisone. In 1 subject ACTH was found to increase the adrenal output whereas aspirin, ascorbic acid, and Pyribenzamine had little, if any, effect.

The possible application of the approach to the measurement of the formation of other substances in the body has been noted.

REFERENCES

1. Anderson, E., Haymaker, W., and Joseph, M., *Endocrinol.* **23**, 398 (1938).
2. Porter, C. C., and Silber, R. H., *J. Biol. Chem.* **185**, 201 (1950).
3. Perkoff, G. T., Sandberg, A. A., Nelson, D. H., and Tyler, F. H., *A.M.A. Arch. Int. Med.* **92**, 1 (1954).

4. Liddle, G. W., Island, D., Renfret, A. P., and Forsham, P. H., *J. Clin. Endocrinol. and Metabolism* **14**, 839 (1954).
5. Silber, R. H., and Porter, C. C., *J. Biol. Chem.* **210**, 923 (1954).
6. Silber, R. H., Yamamoto, R., and Chow, B. F., to be published.
7. Eckert, C., Weichselbaum, T. E., *et al.*, personal communication, 1954.
8. Hume, D. M. and Nelson, D. H., *Fed. Proc.* **13**, 73 (1954).
9. Silber, R. H. and Morgan, E., to be published.
10. Peterson, R. E., and Wyngaarden, J. B., *Ann. N. Y. Acad. Sci.* **61**, 297 (1955).

Quantitative Identification of Urinary Calculi

Reid H. Leonard and Arthur J. Butt

THE USUAL METHODS of identification of urinary calculi are based upon qualitative chemical reactions (1, 2, 3). These methods are difficult to interpret and easily lead to erroneous conclusions. Other, more specific, methods are based upon mineralogic technics, x-ray patterns, and infrared absorption curves (4, 5) but are of limited use because of the infrequent availability of the required instrumentation.

Calculi may be identified by quantitative methods in regular clinical use. Most specimens may be identified by determination of calcium, phosphorus, and oxalic acid, while the less frequent types may require a determination of ammonia and soluble nitrogen. In addition to the quantitative determinations, 2 observations and 4 qualitative tests assure accuracy in identification of calculi.

The compounds found in calculi are well defined with the exception of the phosphates. Table 1 gives the analytical compositions of the compounds which are ordinarily found in calculi.

PROCEDURE

Preparation

The sample should be free of tissue, blood, lint, and such extraneous matter. It is dried in a vacuum for 15 minutes in fragments weighing not over 100 mg. placed in a tube immersed in boiling water. Small calculi may be weighed whole but larger ones should be ground to a powder before sampling. With large laminated calculi it is clinically important to separate the nucleus and portions of predominating layers and analyze them separately. About 10 mg. is sufficient for analysis, although with care as little as 1 mg. may be used.

The weighed specimen is placed in a tube and 1–2 ml. of 1:4 hydrochloric acid (HCl) is added. Solution takes place, sometimes slowly, at or near the boiling point. Observation of copious evolution of gas indi-

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Table 1. ANALYTICAL COMPOSITION OF COMPOUNDS FOUND IN CALCULI

Compound	*Calcium* (%)	*Phosphorus* (%)	*Oxalic acid* (%)	*Other*
Calcium oxalate hydrate	27.4	0.0	61.6	..
Calcium oxalate dihydrate	24.4	0.0	54.8	..
Dicalcium phosphate dihydrate	23.3	18.0	0.0	..
Carbonate-apatite	38.8	18.0	0.0	carbon dioxide
Hydroxy-apatite	39.3	18.3	0.0	..
Calcium carbonate	40.0	0.0	0.0	carbon dioxide
Magnesium ammonium phosphate hexahydrate	0.0	12.6	0.0	5.7% ammonia nitrogen 9.9% magnesium
Uric acid	0.0	0.0	130.[a]	33.3% alkali soluble nitrogen
Cystine	0.0	0.0	150.[a]	11.7% nitrogen, soluble in 1:4 HCl
Protein	0.0	0.0	0.0	16% insoluble nitrogen

[a] Calculated from the permanganate consumed.

cates carbonate and should be recorded. A second observation of the relative amount and apparent nature of the insoluble residue is made. Since uric acid and protein do not dissolve, they are removed by centrifugation at this point. The supernatant is analyzed as any acid-soluble specimen, while the residue (a) is dissolved in alkali (e.g., 5N potassium hydroxide [KOH] or 2.5N sodium hydroxide [NaOH]) if it is uric acid or it (b) may be digested and the nitrogen content of the digest determined. After the calculus is dissolved it is diluted to a definite volume in either a calibrated tube, cylinder, or flask. A final dilution to give approximately 1 mg. of calculus per 1 ml. solution is convenient.

Calcium Determination

An aliquot containing about 0.5 mg. of calculus is diluted to 5 ml. with water and isopropanol in such a way that the final solution is 40% alcohol. The calcium is read with appropriate alcoholic standards on a Model B Beckman flame photometer. On small specimens 0.1 mg. will suffice for this determination. Although use of the flame photometer is particularly expedient other methods may be applied. The calcium content for a 1 ml. aliquot (about 1 mg. of calculus) ranges from 0 to 4 times the calcium content of 1 ml. of serum.

Phosphorus Determination

An aliquot representing about 0.2 mg. of calculus is analyzed by the method of Fiske and SubbaRow (6).

Oxalic Acid Determination

An aliquot representing 1–5 mg. of calculus is acidified with 2 ml. of 2N sulfuric acid (H_2SO_4), a drop of 5% manganese sulfate ($MnSO_4$) is added, and after warming the mixture is titrated rapidly with 0.010N potassium permanganate ($KMnO_4$). Most samples give sharp endpoints; however, both uric acid and cystine react and give indefinite and fading endpoints. Uric acid calculi give a brown color. One milliliter of 0.010N $KMnO_4$ is equivalent to 0.45 mg. of oxalic acid, about 0.6 mg. of uric acid, and about 0.7 mg. of cystine. Consequently the presence or absence of uric acid and cystine must be determined by qualitative or quantitative reactions on every specimen which shows a significant permanganate consumption.

Auxiliary Determinations

A qualitative test for ammonia is made with Nessler's reagent added by drops to a small portion of the solution. If much ammonia is shown to be present then this constituent is analyzed quantitatively; however, the presence of calcium will require separation of the ammonia either by distillation or diffusion before it can be quantitatively determined. Magnesium ammonium phosphate usually can be determined by direct nesslerization. Insoluble residues, alkali-soluble calculi, and those not containing much calcium and phosphorus are digested with concentrated H_2SO_4 and potassium persulfate ($K_2S_2O_8$). After dilution of the digest a portion is used for the nitrogen determination. Magnesium is detectable by flame photometry or by the color reaction with *p*-nitrobenzene-azo-resorcinol. Uric acid is detected and determined by the method of Brown (7). Cystine is detected by the nitroprusside reaction (8).

Although this particular selection of procedures is used in our laboratory, the specimen solution may be analyzed by adaptation of methods routinely used for calcium, phosphorus, nitrogen, and uric acid.

INTERPRETATION OF RESULTS

For purposes of interpretation, urinary calculi have been placed in 4 groups: oxalate, phosphate, mixed oxalate-phosphate, and nitrogen-containing.

Oxalate Calculi

The oxalate calculi are the most common type and are simple to identify. Some representative analyses and interpretations are given in Table 2. Oxalate types do not evolve carbon dioxide upon solution in acid;

Table 2. Analysis of Oxalate-type Calculi

#	Found			Computed			
						Calcium oxalate dihydrate	
	Calcium (%)	Oxalic acid (%)	Phosphorus (%)	Calculated phosphate (%)	Corrected calcium (%)	on calcium (%)	on oxalic acid (%)
130	23.8	51.7	1.0	5.6	22.5	92	94
157	24.1	55.0	0.5	2.8	23.5	96	100
164	25.2	54.0	0.25	1.4	24.9	102	99
199	26.7	57.8	0.5	2.8	26.1	107	106
275	24.9	53.0	0.37	2.1	24.4	100	97
312	26.6	55.8	0.59	3.3	25.8	106	102
336	25.0	51.0	0.9	5.0	23.8	98	93
338	28.6	53.4	0.36	2.0	28.1	115	97
351	24.8	54.6	0.29	1.6	24.4	100	100
418	27.2	60.8	0.3	1.7	26.8	110	111
471	28.4	57.9	0.11	0.6	28.3	116	106

a trace of protein or material containing insoluble nitrogen is usually left after solution; a faint test for ammonia is usually found; uric acid and cystine are usually absent or present in trace amounts; and magnesium is absent. Small amounts of phosphorus are usually found.

In order to calculate the composition, it is first necessary to correct the total calcium figure for that which is combined with phosphorus. To do this, the percentage of phosphorus is multiplied by 1.29 and the result subtracted from the total calcium. The calculation of calculi components is made on the basis of the composition given in Table 1. Calcium oxalate as the di- or monohydrate form may then be calculated from both the corrected calcium and the oxalic acid. The examples given in Table 2 are all as the dihydrate form. The agreement between the two methods of calculation is good and serves as evidence of the validity of the oxalic acid determination. When calcium oxalate monohydrate is calculated as calcium oxalate dihydrate, a value of 112.5 per cent is obtained. Oxalate calculi which figure more than 100 per cent as the dihydrate probably contain much monohydrate material. Examples 418 and 471 are apparently almost entirely the monohydrate form. Example 338 shows a much higher result when based on the calcium content than when based on the oxalic acid. We have encountered other specimens which exhibited this high calcium content for an oxalate-type calculus. The nature of the binding anion in these cases is not known.

Phosphate Calculi

The phosphate calculi have been reported to contain several different calcium phosphate salts (4). The usual phosphate specimen contains

Table 3. Analysis of Phosphate-type Calculi

	Found				Computed			
							Computed from calcium	
#	Calcium (%)	Oxalic acid (%)	Phosphorus (%)	Carbon[a] dioxide	Calculated phosphate (%)	Calcium: phosphorus ratio	as apatites (%)	as dicalcium phosphate (%)
156	27.8	3.4	16.8	0	93	1.56	68	113
184	21.1	3.6	16.0	0	89	1.22	50	84
187	19.0	4.5	16.4	P	91	1.04	44	73
204	23.9	2.6	16.2	P	90	1.40	59	97
265	26.9	2.1	14.9	P	83	1.74	67	112
268*	25.4	3.2	19.0	P	105	1.26	62	103
299	33.5	8.3	15.6	P	87	1.91	77	128
335	25.6	5.2	13.3	P	74	1.75	60	100
354	29.2	4.8	16.3	0	91	1.66	70	116
364	22.1	2.2	13.1	P	73	1.61	54	91
436	26.0	2.1	13.3	P	74	1.89	65	108
458	19.1	2.1	14.4	P	80	1.26	47	78

[a] #268 is a calculus from prostate. P = present, 0 = absent.

some carbonate; traces of protein; no uric acid, cystine, or magnesium; and small amounts of oxalic acid. The total calcium may be corrected for calcium oxalate by deducting 0.45, the oxalic acid percentage. Table 1 shows the similar phosphorus contents of apatites and dicalcium phosphate. Examples of the phosphate-type calculi are given in Table 3. Since the apatites and dicalcium phosphate dihydrate calculate the same on the basis of phosphorus, this figure is given in Table 3 under the column heading "Calculated phosphate." The weight ratio of calcium to phosphorus has been useful as a guide to the nature of the calcium phosphate salts in calculi. The ratios for carbonate-apatite, hydroxyapatite, and dicalcium phosphate are 2.15, 2.15, and 1.29, respectively. The ratios shown in Table 3 are made after the calcium has been corrected for oxalate. It is possible to calculate from the ratio any proportion of two out of the possible salts. However, for clinical purposes it is sufficient to know that a "calcium phosphate" is the calculus component, although for investigative purposes the nature of the calcium phosphate is important.

Infrared spectroscopy strongly suggests an apatite-like crystal structure even in those instances where the ratio of calcium to phosphorus is considerably below that required for an apatite (5). The ratios obtained show a composition that usually falls between dicalcium phosphate and the apatites. In order to more clearly demonstrate this, the apatite and dicalcium phosphate dihydrate contents based upon the calcium found are given in Table 3. The apatite values are uniformly low while the

Table 4. Analysis of Mixed Oxalate-Phosphate Calculi

	Found				Computed			
							Calcium in products as % of Ca found when based on	
#	Calcium (%)	Oxalic acid (%)	Phosphorus (%)	Carbon dioxide	Calcium oxalate dihydrate (%)	Calculated phosphate (%)	Dicalcium phosphate (%)	Apatites (%)
134	26.8	27.0	9.3	0	49	52	90	120
143	27.5	21.2	11.9	0	39	66	90	127
163	27.0	13.5	14.3	P	25	79	90	136
227	32.2	35.1	6.6	0	64	37	75	93
235	25.3	31.0	6.8	P	57	38	89	113
239	28.5	39.5	4.8	0	72	27	84	98
246	29.0	13.9	12.5	P	25	69	77	113
258	33.0	31.0	8.2	0	57	46	74	96
309	28.6	9.5	14.6	P	17	81	80	124
337	36.1	41.7	4.3	0	76	24	67	77

P = present; 0 = absent.

dicalcium phosphate values are frequently much too high. The large variability of the phosphate-type calculi precludes any simple but precise means of expressing their composition. It is not yet known how much significance can be attached to the calcium:phosphorus ratio.

Mixed Oxalate-Phosphate Calculi

The mixed oxalate-phosphate types are calculated on the basis of oxalic acid to calcium oxalate dihydrate and on the basis of phosphorus to "calculated phosphate." Examples are given in Table 4. The sum of the two calculated salts is within 5 per cent of a complete accounting of the calculi weights. However, when the calcium contents of the calculated salts are compared to the calcium determined by analysis there is poor correlation. Table 4 gives the calculated calcium sum or recovery as percentage of the calcium found. These recovery figures are consistently low when the composition is on the basis of dicalcium phosphate dihydrate and generally high when calculated on the basis of carbonate apatite. The results are adequate to classify the calculi for clinical significance.

Nitrogen-Containing Calculi

Examples of nitrogen-containing calculi are given in Table 5. When a high oxalic acid value but a low or absent calcium value is found then either uric acid or cystine should be suspected. A determination of soluble nitrogen, aided by qualitative tests, will establish which of the

Table 5. Analysis of Nitrogen-containing Calculi

	Found									Computed
#	Calcium (%)	Oxalic acid (%)	Phosphorus (%)	Carbon dioxide	Soluble nitrogen (%)	Uric acid (%)	Ammonia nitrogen (%)	Cystine (%)	Magnesium (%)	Composition
95	0.3	70	0.0	0	12.2	0	1.0	P	0	96% cystine
166	10.2	1.8	14.1	0	0	0	P	0	8	81% magnesium ammonium phosphate; 22% calcium phosphates
175	0.0	60	0.0	0	31.5	87.5	0	0	0	87% uric acid
182	0.6	129	0.0	0	12.0	0	0	P	0	103% cystine
183	0.0	73	0.0	0	30.6	P	0	0	0	92% uric acid
192	0.0	135	0.6	P	12.1	P	0	86	0	86% cystine
279	1.9	45	0.0	0	..	80	0	0	0	80% uric acid; 8% calcium oxalate ?
437	2.8	60	0.1	0	..	67	0	0	0	67% uric acid; 11% calcium oxalate ?
451	8.6	1.7	13.2	P	..	0	4.4	0	P	77% magnesium ammonium phosphate; 19% calcium phosphates
52	14.9	1.8	13.0	P	..	0	4.2	0	P	74% magnesium ammonium phosphate; 21% calcium phosphates; 17% calcium carbonate ?

P = present; 0 = absent.

two is present. Since uric acid is insoluble in 1:4 HCl, while cystine is soluble, the distinction between these two components is frequently known from the start. Quantitative determination of either uric acid or cystine is used in cases where there is still doubt as to the major component of the calculus. Magnesium ammonium phosphate can be identified by the determination of either ammonia nitrogen or of magnesium.

DISCUSSION

Determination of the major component of a calculus is usually adequate for clinical purposes but cases of mixed composition sometimes require the differentiation of components occurring to the extent of only about 10 per cent. It is frequently possible to remove a nucleus from a laminated calculus as well as samples from different layers. Calculi which exhibit marked lamination are apt to differ in composition. The nuclei and small embryonic specimens, of less than 20 mg., are of much diagnostic value since they frequently point to the underlying cause of the calculus forma-

tion. The procedure as outlined has been able to identify all specimens submitted, except for 1 prostatic calculus. In 2 instances inert material has been encountered in a portion of the specimen.

The distinction between the 2 hydrates of calcium oxalate is primarily theoretical and is thought to be related to the hydration of the patient. The calcium:phosphorus ratio is important from the etiologic viewpoint and may be helpful in treating some cases. The proportion of oxalate to phosphate is of theoretical and therapeutic importance.

Few specimens are apt to give deceptive results. These are clarified by performing the qualitative tests routinely with the quantitative determinations. Deceptive conditions are produced by the presence of calcium compounds with magnesium ammonium phosphate, calcium oxalate with uric acid, and calcium phosphate with cystine.

SUMMARY

Quantitative determination of calcium, phosphorus, and oxalic acid, assisted by occasional determination of ammonia nitrogen and insoluble nitrogen when indicated by qualitative tests, provides a means of computing the component substances of calculi. The determinations may be performed by slight modifications of the methods in use in the routine clinical chemistry laboratory, although flame photometry is particularly convenient for the determination of calcium.

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REFERENCES

1. Higgins, C. C., *Renal Lithiasis*, Springfield, Ill., Thomas (1944), p. 53.
2. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, (ed. 13) Philadelphia, Blakiston (1954), p. 854.
3. Hepler, O. E., *Manual of Clinical Laboratory Methods*, (ed. 4), Springfield, Ill., Thomas (1949), p. 16.
4. Prien, E. L., and Frondel, C., *J. Urol.* **57**, 949 (1947).
5. Beischer, D. E., *J. Urol.* **73**, 653 (1955).
6. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.* **66**, 375 (1925).
7. Brown, H., *J. Biol. Chem.* **158**, 601 (1945).
8. Block, R. J., and Bolling, D., *Amino Acid Composition of Proteins and Foods* (ed. 1), Springfield, Ill., Thomas (1945), p. 164.

Calcium Determination in Biologic Material

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THE CLASSICAL PROCEDURE for the determination of calcium in biologic fluids involves the precipitation of calcium as the oxalate and titration of the oxalate with potassium permanganate. The permanganate titration has certain difficulties. The oxalate solution has to be heated during the titration (1) and the end point is unreliable in dilute solutions (2). The use of cerate oxidimetry avoids these circumstances. The principles of cerate oxidimetry have been well established in the chemical literature and there have been numerous applications to the determination of calcium (2–8). The present report defines conditions which proved to be most satisfactory for the determination of calcium in biologic material. The method has proved reliable through several years of use since 1940 in this and other laboratories. The accuracy is ±2 per cent as measured by the recovery of known amounts of calcium.

PRINCIPLE OF METHOD

Calcium is precipitated as the oxalate salt which is then oxidized by ammonium hexanitratocerate (9). The excess cerate is titrated with ferrous ion, using ortho-phenanthroline as an oxidation-reduction indicator.

REAGENTS

(1) 0.4% aqueous solution phenol red.
(2) 4N ammonium hydroxide.
(3) 10% glacial acetic acid.
(4) 4% ammonium oxalate.

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(5) Wash solution (10): Equal parts of 95% ethanol, ethyl ether, and water containing 2% ammonium hydroxide. Prepare daily.
(6) 2N sulfuric acid.
(7) 0.014M ammonium hexanitratocerate in 2N sulfuric acid. 7.67 Gm. of ammonium hexanitratocerate is dissolved in liter of 2N H_2SO_4.
(8) 0.025M *o*-phenanthroline ferrous sulfate ("Ferroin").[1] Dilute with water to 0.005M for use.
(9) 0.1M ferrous ammonium sulfate stock solution. 19.6 Gm. of ferrous ammonium sulfate is dissolved in 500 ml. 0.36N H_2SO_4. Stock solution is diluted with water to 0.005M for use.
(10) Standard 0.01N sodium oxalate. To 0.6698 Gm. of sodium oxalate dissolved in distilled water is added 5 ml. of conc. H_2SO_4 and the solution diluted to 1 liter (11).
(11) 4N sulfuric acid.

METHODS

Serum or Heparinized Plasma

In a 15 ml. conical centrifuge tube 2 ml. of serum or heparinized plasma is mixed with 2 ml. of distilled water; 1 ml. of 4% ammonium oxalate is added. The mixture is allowed to stand about 12 hours or if more convenient, overnight.

The sample is centrifuged at 2500 rpm for 15 minutes and the supernatant fluid removed by aspiration, using a fine-tipped pipet. The precipitate is washed with 3 ml. of wash solution. To insure thorough removal of excess oxalate, the wash solution is added slowly along the side of the tube. The precipitate is stirred with a thin glass rod and the rod is washed with wash solution as it is removed from the sample. The sample is centrifuged for 10 minutes at 2500 rpm. The supernatant wash is carefully decanted and the tube allowed to drain for a few minutes while resting inverted on absorbent paper. The washing procedure is repeated.

The moist calcium oxalate precipitate is dried by placing the tube in a water bath at room temperature and bringing the bath slowly to a boil. To the dried oxalate 2 ml. of 2N H_2SO_4 is added and the tube is heated for 1 minute to bring all the precipitate into solution. The tube is cooled to room temperature and 1 ml. of 0.014M cerate is added. The sample is stirred and allowed to stand for 30 minutes, with the stirring rod left in the tube. Orthophenanthroline indicator, 0.02 ml. of 0.005M, is added and the excess cerate is titrated with 0.005M ferrous ammonium sulfate,

[1] This oxidation-reduction indicator is purchased as an 0.025M solution.

using a 5 ml. microburet. The color change is from yellow to colorless, to a faint blue, and then to salmon pink, which represents the end point. The end point is quite sharp and distinct. Only 0.002 ml. of 0.005M ferrous ammonium sulfate is needed to effect the color change.[2, 3]

Urine

One to three milliliters of urine is pipetted into a 15 ml. conical centrifuge tube. The *p*H is adjusted to 6.5 by adding 1 drop of phenol red indicator solution and 4N NH_4OH by drops until the solution is pink. Ten per cent glacial acetic acid is then added by drops until the solution turns yellow. The calcium is precipitated by adding 1 ml. of 4% ammonium oxalate.[4] The solution is mixed and allowed to stand. After precipitation of the calcium oxalate the procedure is followed as described for plasma.

Dried Tissues and Feces

A sample of about 1 Gm. is placed in a platinum crucible (about 10 ml. capacity) with enough concentrated sulfuric acid to cover the specimen. The sample is dried at 100° and then ashed in a muffle furnace at 700° for 12 hours. The white ash is dissolved in 1–3 ml. of concentrated hydrochloric acid and transferred to a 25 ml. volumetric flask with hot water. The sample is allowed to cool and then diluted to 25 ml. A sample containing about 10 microequivalents of calcium is placed in a 15 ml. conical centrifuge tube, and the *p*H is adjusted to 6.5 and the calcium precipitated following the procedure detailed for the determination of calcium in urine.

STANDARDIZATION

The ferrous ammonium sulfate is standardized against 0.01N sodium oxalate and the cerate is standardized against the iron. One milliliter of

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[2] At the time the cerate is added to the oxalate, there should be sufficient excess cerate to impart some yellow color to the solution. If the solution turns colorless, indicating that there is more than 14 microequivalents of calcium present, more cerate is added so that some yellow color persists.

[3] Do not use Tygon tubing as part of the titration apparatus, as this tubing oxidizes the ferrous iron.

[4] The precipitate of 10 microequivalents of calcium oxalate just about covers the bottom of a 15 ml. conical centrifuge tube. Should the precipitate appear considerably smaller or larger than this after the addition of ammonium oxalate, it would be advisable to alter the amount of the sample. One ml. of 0.014N cerate will be sufficient to titrate 14 microequivalents of calcium. If there is more calcium present than 14 microequivalents, more cerate may be added at the time of titration, but it becomes inconvenient to add much more than 5 ml. of cerate to a 15 ml. centrifuge tube.

0.014M cerate is added to 1 ml. of 0.01N sodium oxalate plus 1 ml. of 4N sulfuric acid. The solution is allowed to stand for 30 minutes. Orthophenanthroline, 0.02 ml. of 0.005M, is added and the solution is titrated against the iron solution. One milliliter of cerate plus 2 ml. of 2N sulfuric acid are also titrated directly against the iron without the addition of oxalate. The titration difference between the cerate with oxalate and cerate without oxalate represents the amount of iron which is equivalent to 1 ml. of 0.01N oxalate, which in turn is equivalent to 0.2 mg. of calcium.

SUMMARY

A method has been described for the determination of calcium in plasma, urine, and tissue and feces. Calcium is precipitated as the oxalate salt and the oxalate is determined by cerate oxidimetry.

REFERENCES

1. Clark, E. P., and Collip, J. B., *J. Biol. Chem.* **63,** 461 (1925).
2. Kirk, P. L., and Tompkins, P. C., *Ind. Eng. Chem., Anal. Ed.* **13,** 277 (1941).
3. Katzman, E., and Jacobi, M., *J. Biol. Chem.* **118,** 539 (1937).
4. Ellis, G. H., *Ind. Eng. Chem., Anal. Ed.* **10,** 112 (1938).
5. Larson, C. E., and Greenberg, D. M., *J. Biol. Chem.* **123,** 199 (1938).
6. Kochakian, C. D., and Fox, R. P., *Ind. Eng. Chem., Anal. Ed.* **16,** 762 (1944).
7. Smith, W. R., *Analyst* **69,** 14 (1944).
8. Wheatly, V. R., *Analyst* **69,** 207 (1944).
9. Smith, G. F., Sullivan, V. R., and Frank, G., *Ind. Eng. Chem., Anal. Ed.* **8,** 449 (1936).
10. Wang, C. C., *J. Biol. Chem.* **111,** 443 (1935).
11. Hawk, P. B., and Bergeim, O., *Practical Physiological Chemistry* (ed. 11). Philadelphia, Blakiston, 1937, p. 469.

Serum Beta-Lipoproteins of Normal, Atherosclerotic, and Lipemic Individuals

Changes in Concentration, Stability, and Mobility upon Incubation at 6.5° and 37.5°, as Determined by Paper Electrophoresis

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IT HAS BEEN OBSERVED by many investigators that the serum lipoproteins tend to decrease when allowed to stand for any length of time at room temperature or at refrigerator temperature. The latter, together with the observations of Horlick (1) who reported that the serum lipoproteins of a person with atherosclerosis were less stable when incubated with *Clostridium welchii* lecithinase than the serum lipoproteins of a normal individual, prompted us to study any changes in the concentration and stability of the serum of normal, lipemic, and atherosclerotic individuals upon incubation at 6.5° and 37.5°.

Hoch and Chanutin (2) reported that in serum stored at room temperature, a portion of the β-globulin, as measured by a Tiselius electrophoretic apparatus, increased its mobility within 36 hours of storage. Since the β-lipoproteins appear to be associated with the β-globulin fraction as far as mobility is concerned, it was decided to determine whether β-lipoproteins of normal, lipemic, and atherosclerotic sera would show a similar change in mobility on standing.

METHODS

Clotted blood was obtained from 8 normal, 8 lipemic, and 7 atherosclerotic individuals. The serum was separated as quickly as possible,

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and a lipoprotein pattern was run. The sera were allowed to stand for 24, 48, and 72 hours in a refrigerator at 6.5° and in a water bath at 37°. The appearance of a turbidity in the serum was noted, and lipoprotein patterns were obtained every 24 hours. In some cases, serum protein patterns were run along with the lipoproteins.

Lipoprotein and protein curves were obtained by a method previously described (3). This consisted in streaking filter paper with 0.04 ml. and 0.01 ml. serum, for a lipoprotein and protein separation respectively, and running for 24 hours in an Ivan Sorvall apparatus at 360 V. (4–6 ma.) in a barbiturate buffer at a *p*H of 8.55. The lipids were visualized by staining with Sudan III and the proteins by Amido Black B. The density of the stains was obtained by means of a Photovolt densitometer using no filter. The curves were plotted and the position of the peaks noted in inches from the origin. The concentration of the beta lipoproteins was expressed as square inches of area under the respective curves as measured by a planimeter.

A normal individual was defined as any person under 55 years of age who had no organic disturbances at the time, whose serum lipids were between 350 and 800 mg./100 ml. and whose serum cholesterol was between 150 and 300 mg./100 ml. Serum lipids were determined gravimetrically by a modification of the method of Bloor (4, 5) while the serum cholesterol was determined by the method of Kaye (6). A lipemic individual was one under 55 years of age who had no previous history of coronary involvement, but whose serum lipids were either greater than 800 mg./100 ml. or whose serum cholesterol was more than 300 mg./100 ml. Atherosclerotic individuals were chosen according to criteria set forth in a previous publication (7).

RESULTS AND DISCUSSION

In Tables 1, 2, and 3 are listed the concentration and mobility of the β-lipoproteins of normal, atherosclerotic, and lipemic serum freshly drawn and after incubating at either 6.5° or 37° for 24, 48, and 72 hours. An asterisk denotes the appearance of a visible turbidity in the serum upon incubation. In such cases, a repeat study was performed. Generally, the concentration of the β-lipoproteins tended to decrease upon standing, the decrease being practically the same for normal, atherosclerotic, or lipemic serum, and being independent of the temperatures at which the serum was incubated.

The average results denote that at either 6.5° or 37°, standing for 24 hours resulted in a decrease in the β-lipoproteins of about 10 per cent,

Table 1. β-LIPOPROTEIN STUDIES ON NORMAL SERUM

Patient	Total cholesterol (mg./100 ml.)	Total lipids (mg./100 ml.)	Concentration of β-lipoprotein (in.³/0.04 ml.)							Mobility of β-lipoprotein peak (inches from origin)						
			Fresh serum	24-Hour incubation		48-Hour incubation		72-Hour incubation		Fresh serum	24-Hour incubation		48-Hour incubation		72-Hour incubation	
				6.5°	37°	6.5°	37°	6.5°	37°		6.5°	37°	6.5°	37°	6.5°	37°
1	197	578	2.64	2.23	2.34	1.85	2.13	1.51	1.64	2.38	1.75	2.38	1.88	2.36	1.88	2.25
2	208	510	2.54	2.17	2.68	1.76	1.64	1.74	1.23	2.38	1.63	2.13	2.00	2.38	1.75	2.38
3	165	605	2.43	1.99	2.14	1.58	2.11	1.73	1.72	2.13	1.63	2.38	1.00	2.25	1.25	2.50
4	230	700	2.36	2.07	2.23	2.07	1.88	2.14	1.82*	1.88	1.13	2.25	1.00	2.25	0.75	0.38*
	235	747	2.24	1.95	1.93	1.83	1.85	1.95	1.92	1.88	2.25	2.75	1.63	2.50	2.00	..
5	193	580	2.29	2.03	1.76	1.97	1.92	1.94	2.07*	1.88	1.25	1.50	1.63	2.25	1.25	0.50*
	..	..	2.09	1.92	1.78	1.71	1.58*	1.65	1.81*	2.13	1.50	2.13	2.38	2.00*	1.75	0.0*
6	157	570	1.94	1.74	1.64	1.43	1.30	1.45	1.03	1.88	1.88	2.63	2.50	2.38	2.13	1.38
7	153	700	2.67	2.68	2.56	2.20	2.12	2.23	2.56	1.75	2.00	2.38	1.88	..	1.38	..
8	188	587	1.94	1.74	1.63*	1.80	1.63*	1.67	1.43*	2.13	1.88	1.00*	1.88	0.25*	1.00	0.0*
AVERAGE	..	..	2.31	2.05	2.07	1.82	1.82	1.80	1.72	..	..	..	..	..	..	..

* Denotes a turbid serum.

Table 2. β-LIPOPROTEIN STUDIES ON ATHEROSCLEROTIC SERUM

Patient	Total cholesterol (mg./100 ml.)	Total lipids (mg./100 ml.)	Concentration of β-lipoprotein (in.²/0.04 ml.)							Mobility of β-lipoprotein peak (inches from origin)						
			Fresh serum	24-Hour incubation		48-Hour incubation		72-Hour incubation		Fresh serum	24-Hour incubation		48-Hour incubation		72-Hour incubation	
				6.5°	37°	6.5°	37°	6.5°	37°		6.5°	37°	6.5°	37°	6.5°	37°
1	267	980	2.37	2.36	2.34	2.13	2.33	1.43	1.76	2.25	2.25	2.75	2.00	2.38	2.13	3.00
2	295	900	2.40	2.20	2.10	2.08	1.97*	2.01	2.03*	2.50	2.25	2.50	2.00	0*	2.00	0*
	357	973	2.44	2.33	2.21	2.23	2.01	1.78	1.82	1.80	1.13	2.38	2.00	2.63	2.13	2.88
3	237	950	2.57	2.12	2.06	2.21	1.80*	2.01	1.65*	2.63	2.00	2.75	2.25	0.38*	2.38	0*
	254	847	2.63	2.42	2.30	2.20	2.12*	2.29	2.22*	2.00	2.00	2.75	1.88	1.13*	1.63	0.25*
4	256	880	1.95	1.66	1.86*	..	2.08*	1.65	1.64*	2.50	1.75	0.25*	..	0*	2.13	0*
	245	963	2.23	2.09	2.02	1.90	1.58*	1.86	1.64*	1.63	1.75	1.75	1.50	1.13*	2.00	0*
5	267	933	2.62	2.17	2.22	2.08	1.77*	1.57	1.41*	2.38	1.88	2.75	1.88	0.38*	1.88	0.38*
	228	933	2.43	2.29	2.24	1.91	2.03	1.84	1.81*	2.00	1.88	2.75	2.25	2.63	1.63	1.50*
6	290	1062	1.90	1.61	2.02	1.90	2.03	1.43	1.72	2.00	2.13	2.75	2.00	2.25	2.25	2.87
7	292	850	2.73	2.14	2.32	1.97	1.99	1.72	1.96	1.80	1.25	2.25	1.75	2.50	1.88	2.50
8	284	950	2.19	1.97	2.00	1.85	2.11	1.63	..	1.88	1.88	2.25	1.63	2.38	2.00	..
AVERAGE	..	..	2.37	2.11	2.14	2.04	1.98	1.77	1.79	..	..	..	..	..	..	..

*Denotes a turbid serum.

Table 3. β-LIPOPROTEIN STUDIES ON LIPEMIC SERUM

Patient	Total cholesterol (mg./100 ml.)	Total lipids (mg./100 ml.)	Concentration of β-lipoprotein (in.²/0.04 ml.)							Mobility of β-lipoprotein peak (inches from origin)						
			Fresh serum	24-Hour incubation		48-Hour incubation		72-Hour incubation		Fresh serum	24-Hour incubation		48-Hour incubation		72-Hour incubation	
				6.5°	37°	6.5°	37°	6.5°	37°		6.5°	37°	6.5°	37°	6.5°	37°
1	273	935	2.75	2.01	1.84	2.21	1.76	1.60	1.63	1.88	2.00	2.38	2.00	2.88	2.00	2.88
2	312	938	2.51	2.18	2.27	2.09	2.22	1.89	2.16	2.50	1.88	2.50	1.88	2.13	2.13	2.75
3	256	843	2.49	2.31	2.14	1.79	2.17	1.78	2.05	2.25	2.00	2.38	1.88	2.38	1.13	2.75
4	280	873	2.50	2.28	2.38	2.18	1.92*	2.09	1.87*	2.25	2.25	2.63	1.75	1.13*	2.13	0.63*
5	278	910	2.21	1.96	2.06*	1.83	2.02*	..	..	2.25	1.87	1.25*	1.75	0.25*	..	..
	249	920	2.69	2.44	2.54	2.50	2.63*	2.37	2.05*	2.50	2.00	2.75	2.25	2.13*	1.75	0*
	..	..	2.40	2.34	2.41	2.27	2.37	2.21	2.19	1.75	2.25	3.00	2.75	3.50	2.00	2.87
6	280	1273	2.91	2.76	2.70	2.69	2.71	2.34	2.60	2.13	1.13	2.13	1.88	2.38	1.75	2.50
7	220	1050	2.23	2.18	2.18	2.30	1.98	..	..	1.88	1.88	2.38	1.88	2.38	..	..
AVERAGE	..	..	2.53	2.27	2.28	2.21	2.20	2.04	2.08	..	..	..	..	..	..	..

* Denotes a turbid serum.

after 48 hours the decrease was 20 per cent, and after 72 hours, 25 per cent.

If the average results for the β-lipoprotein concentrations in fresh serum are examined, it will be noticed that the results are the same for both normal and atherosclerotic individuals, 2.31 and 2.37 respectively. The atherosclerotic patients studied in this report were all ambulatory, and more or less had returned to their normal routine which had been interrupted by the immobilizing myocardial infarct. The above results are in agreement with those reported by the authors in a recent publication (8). The latter stated that in many cases, soon after an immobilizing infarct, the β-lipoproteins were elevated but tended to become normal as the time from the infarct increased. Table 3, however, shows that the β-lipoproteins in fresh lipemic sera run slightly higher than in fresh normal or atherosclerotic sera, averaging 2.53. None of these lipemic sera showed any marked initial turbidity.

Attention is drawn to the very close agreement between the average results of the β-lipoprotein concentrations of sera incubated at 6.5° and at 37°. The greatest difference is 4.5 per cent.

When the figures for the mobility of the β-lipoprotein peaks are examined before and after incubation, the following observations can be made. If the position of the β-lipoprotein peak of freshly drawn serum is used as a reference point, it will be observed that in practically all cases of normal, atherosclerotic, and lipemic serum incubated at 37° the peak becomes more mobile and migrates further from the origin. This change in mobility is somewhat accentuated as the time of incubation is increased from 24 to 72 hours. However, this phenomena does not occur when the serum becomes turbid. In such cases, the peak becomes less mobile, migrating toward the origin.

If the position of the peaks obtained by incubating at 6.5° are noted, a decrease in the mobility of the β-lipoproteins will be found to occur in most cases, the peak of the freshly drawn serum again being used as the reference. This decrease in mobility does not always become greater as the period of incubation is increased. In many cases it becomes less, and in a few cases the decrease in mobility turns into an increase after incubating 48 or 72 hours. This irregularity in results may be due to an inability to obtain a reproducible peak for the β-lipoproteins. If the tables are examined, it will be noted that the peak of the β-lipoproteins of freshly drawn serum varies from 1.63 to 2.63 inches from the origin. This large spread in the position of the β-lipoprotein peak indicates that the increase in mobility of the β-lipoproteins, which has been reported

to occur after incubating at 37°, might not be real. Those cases which report an increase in mobility of less than 1.00 inch lie within the above reported spread, and cannot be considered significant.

However, since the mobilities of a serum incubated at 6.5 and 37° were always determined in a simultaneous run, the observation that the mobility of the β-lipoprotein peak after incubating at 37° is always greater than the peak obtained after incubating at 6.5° must be considered significant. This holds for either normal, atherosclerotic, or lipemic serum. The only exceptions that occur are upon the appearance of a visible turbidity in the serum. This takes place only upon incubating at 37°, never at 6.5°. In most cases, the turbidity appeared after 48 hours. In only 1 case did it appear as early as after 24 hours. When repeat determinations were performed on those sera in which a turbidity did occur, in 5 of 8 cases a turbidity reoccurred. Although the total number of cases is not large enough, it does appear from Tables 1, 2, and 3 that atherosclerotic serum has the greatest tendency to become turbid upon incubating at 37°.

In order to study more fully the variations in the β-lipoprotein pattern after incubation, serum protein as well as lipoprotein patterns were obtained on 12 normal, atherosclerotic, and lipemic sera according to the procedure already described. Two types of curves were obtained, one in which no turbidity occurred, the other in which a turbidity did occur. Figure 1, *A–E*, is typical of a case in which no turbidity occurred after incubation. They represent the protein and lipoprotein curves in an atherosclerotic serum before and after incubation at 6.5° and 37° for 24 and 48 hours.

The curves reveal that the mobility of the protein fractions remain constant whether incubated at 6.5° or at 37°. Likewise, the β-lipoproteins incubated at 6.5° do not show any change in mobility, the peak lying between the β- and γ-globulins. However, the β-lipoproteins incubated at 37° do reveal an increase in mobility after 24 hours, the peak shifting to between the β- and α_2-globulins. This increase in mobility is somewhat accentuated after incubating for 48 hours. Therefore, it is evident that these curves corroborate the inference from figures reported in Tables 1, 2, and 3—that the mobility of the β-lipoproteins incubated at 37° is greater than the mobility of the β-lipoproteins incubated at 6.5°. In addition, Fig. 1, *A–E*, denotes that incubating at 6.5° probably does not cause a decrease in the mobility of the β-lipoproteins.

Although the mobility of the β-lipoproteins increases upon incubating at 37°, no increase was observed in the mobility of the β-globulins. This

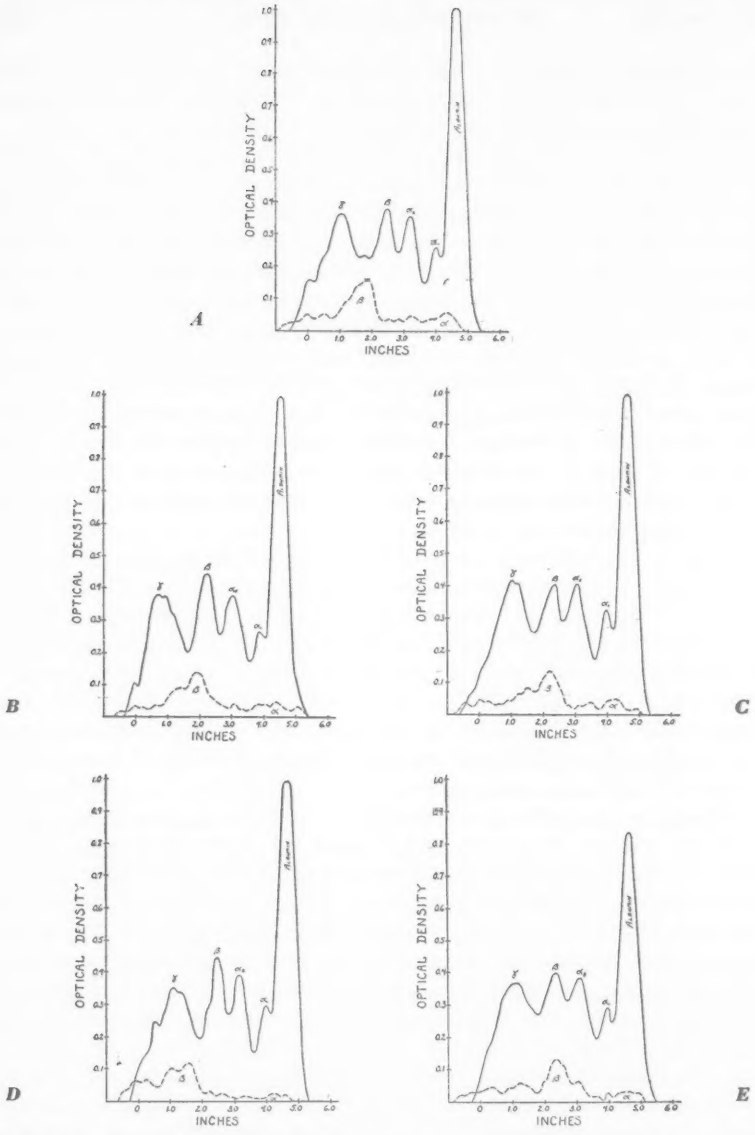

Fig. 1*A*. Protein and lipoprotein fractionation curves of fresh serum in a case of atherosclerosis. Solid lines represent the protein; broken lines the lipid profiles. ***B.*** After 24 hour incubation at 6.5°. ***C.*** After 24 hour incubation at 37.5°. ***D.*** After 48 hour incubation at 6.5°. ***E.*** After 48 hour incubation at 37.5°.

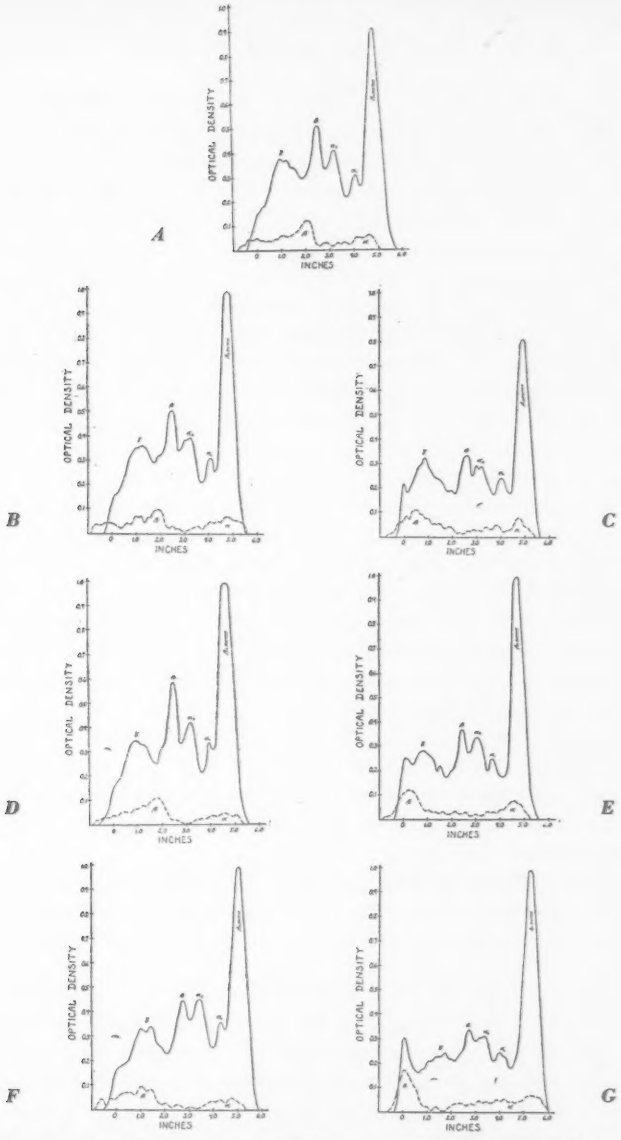

Fig. 2*A*. Protein and lipoprotein fractionation curves of fresh serum from a normal adult. Solid lines represent the protein; broken lines the lipid profiles. ***B***. After 24 hour incubation at 6.5°. ***C***. After 24 hour incubation at 37.5°. ***D***. After 48 hour incubation at 6.5°. ***E***. After 48 hour incubation at 37.5°. ***F***. After 72 hour incubation at 6.5°. ***G***. After 72 hour incubation at 37.5°.

is at variance with the observations of Hoch and Chanutin (2), who stated that using the Tiselius electrophoresis technic, an increase was obtained in the mobility of the β-globulins of stored serum.

Figure 2, *A–G*, is typical of a case in which a turbidity occurred after incubation. They represent the protein and lipoprotein curves in a normal serum before and after incubation at 6.5° and 37° for 24, 48, and 72 hours. It is noticed that at 37° after a period of 24 hours the appearance of a turbidity in the serum is accompanied by a decrease in the mobility of the β-lipoproteins, the peak shifting toward the origin. After 48 hours, when an increase in the turbidity of the serum is evident, it is noticed that the β-lipoprotein peak has moved closer to the origin. At the end of 72 hours, practically the entire β-lipoprotein fraction has concentrated at the origin, denoting an almost complete loss of mobility.

Along with the appearance of a turbidity in the serum is the formation of a sixth protein fraction less mobile than the γ-globulins. As the turbidity increases after incubating for 48 hours, this sixth protein fraction increases at the expense of the β- and γ-globulins, and moves closer to the origin. At 72 hours this new protein fraction has increased even more, and has shifted to such an extent that the peak is located directly at the origin, coinciding with the peak of the β-lipoproteins. The β- and γ-globulins have continued to decrease, and at this point the α-globulins also have begun to decrease.

In the case presented above, the turbidity appeared after only a 24-hour incubation, and was considerable. In other cases where the turbidity does not appear until after 48 or 72 hours and is not marked, this sixth protein fraction is small, and little or no decrease in the other globulins occurs.

The sixth protein fraction is probably "denatured" protein as evidenced by the concomitant decrease in solubility (increase in serum turbidity) and decrease in mobility. Likewise, the β-lipoproteins are probably "denatured" after incubating at 37°, since with increasing turbidity the mobility of the β-lipoproteins decrease considerably.

Two questions arise: first, why the proteins and lipoproteins of some sera should change upon incubation at 37° and some not; second, why a turbidity appears in one serum specimen but not in another, when obtained from the same individual at a later date. These questions may possibly have some bearing upon the pathogenesis of atherosclerosis.

Nothing has been stated concerning the α-lipoproteins, since no marked change in the concentration, stability, and mobility of this fraction after incubation at 6.5° or 37° has been noted, employing the separation and staining technic described in this paper.

SUMMARY

1. Serum lipids, cholesterol, and lipoproteins were determined on 8 normal, 7 atherosclerotic, and 8 lipemic individuals in the fasting state. Serum lipids and cholesterol were determined chemically, the lipoproteins by paper electrophoresis. Serum lipoproteins were also performed after incubating at 6.5° and 37° for 24, 48, and 72 hours, and changes in the physical appearance of the serum, and in the concentration and mobility of the β-lipoproteins were observed. In 12 of the above cases, serum protein patterns were noted with the lipoprotein patterns.

2. In many cases a visible turbidity appeared upon incubating at 37°, never upon incubating at 6.5°. In most cases this turbidity appeared after incubating for 48 hours, and tended to occur more frequently in atherosclerotic sera than in normal sera.

3. The concentration of β-lipoproteins decreased upon incubating either at 6.5° or at 37°, the decrease being of the same order of magnitude for normal, atherosclerotic, or lipemic sera.

4. Incubating at 37° increased the mobility of the β-lipoproteins (except when a turbidity appeared), the peak shifting from between the β- and γ-globulins to between the β- and α_2-globulins. Incubating at 6.5° had no effect on the mobility of the β-lipoproteins.

5. When a turbidity appeared upon incubating at 37°, the mobility of the β-lipoproteins decreased, the peak shifting toward the origin. As the turbidity became marked, a sixth protein fraction appeared, of slower mobility than the γ-globulin. This protein fraction first appeared to form at the expense of the β- and γ-globulins and then at the expense of the α-globulins, all of which decreased. It is considered that the turbidity is due to the formation of "denatured" proteins and lipoproteins which are characterized by a decrease in mobility.

6. No consistent changes were noted in the concentration, stability and mobility of the α-lipoproteins upon incubating at either 6.5° or 37°.

REFERENCES

1. Horlick, L., *Circulation* **10**, 30 (1954).
2. Hoch, H., and Chanutin, A., *J. Biol. Chem.* **209**, 661 (1954).
3. Gottfried, S. P., Pope, R. H., Friedman, N. H., and Di Mauro, S., *J. Lab. Clin. Med.* **44**, 651 (1954).
4. Bloor, W. R., *J. Biol. Chem.* **17**, 377 (1914).
5. Bloor, W. R., *J. Biol. Chem.* **77**, 53 (1928).
6. Kaye, I. A., *J. Lab. Clin. Med.* **25**, 966 (1940).
7. Gottfried, S. P., Friedman, N. H., Pope, R. H., Akerson, I. B., and Di Mauro, S., *Circulation* **10**, 271 (1954).
8. Gottfried, S. P., Pope, R. H., Friedman, N. H., Akerson, I. B., and Di Mauro, S. *Am. J. Med. Sci.* **229**, 34 (1955).

A Note on Quantitative Urobilinogen Determinations

Bernard Balikov

THE WATSON METHOD for the quantitative estimation of urobilinogen in urine and feces in the evaluation of hepatic and hemolytic diseases (1–4) has become widely accepted as a useful tool in clinical practice. The optimum acidity for extraction of urobilinogen from the ferrous hydroxide filtrate, however, has been dealt with vaguely in the literature. Studies on this step of the procedure show it to have a marked influence upon the result.

MATERIAL AND METHOD

The urine specimens were selected from patients under observation for various forms of liver disease and the stool specimens from those received in the laboratory for routine parasitologic or bacteriologic examinations.

The method of Schwartz, Sborov and Watson (1) was employed for the urobilinogen determinations except for the establishment of the standard curve using pontacyl dyes which is described by Watson and Hawkinson (4). Readings were made at 565 mμ on the Coleman Junior Spectrophotometer Model 6A.

The critical aspect of this study is concerned with the concentration of acetic acid in urine or fecal samples after the addition of fresh ferrous sulfate and 10% sodium hydroxide which yields final concentrations of 2.5% sodium hydroxide in urine samples and 2.0% in fecal specimens. At this point varying amounts of water and glacial acetic acid were

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added to the ferrous hydroxide filtrate to make final acid concentrations ranging from 0.1% to 50%. Extraction with petroleum benzine was then carried out in the usual fashion.

RESULTS

Urine

Identical aliquots of ferrous hydroxide filtrate were pipetted into each of several separatory funnels. Water and glacial acetic acid were added to make the final volume 50 ml. and extraction with petroleum benzine, and so on, was carried out in the usual fashion. Results of a series of experiments are shown in Fig. 1. It is apparent that the most complete extraction is realized with a glacial acetic acid concentration of 2%. That this extraction actually is complete was checked by a second extraction made immediately after the first, in which a negligible amount of urobilinogen was recovered (99% transmittance). Similar results were obtained using either 5 or 10 ml. of filtrate for extraction, indicating complete extraction of at least 10 ml. of filtrate.

Determinations of *p*H over the critical range of 0.5 to 4.0% glacial

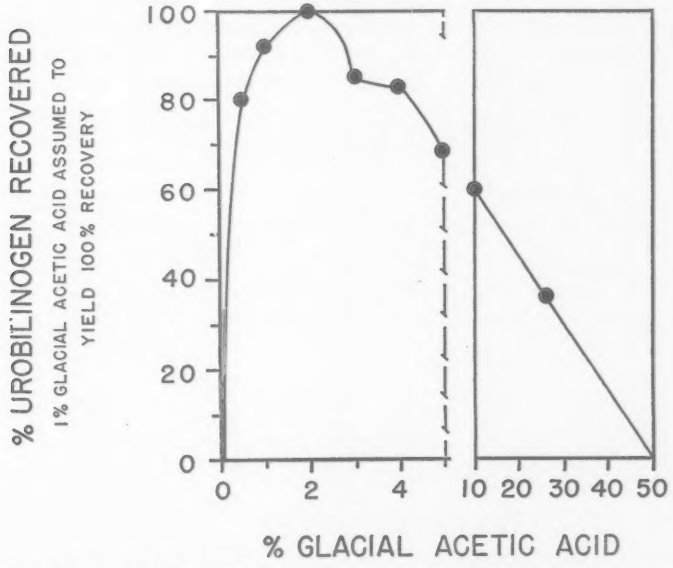

Fig. 1. Recovery of urine urobilinogen from varying glacial acetic acid concentrations.

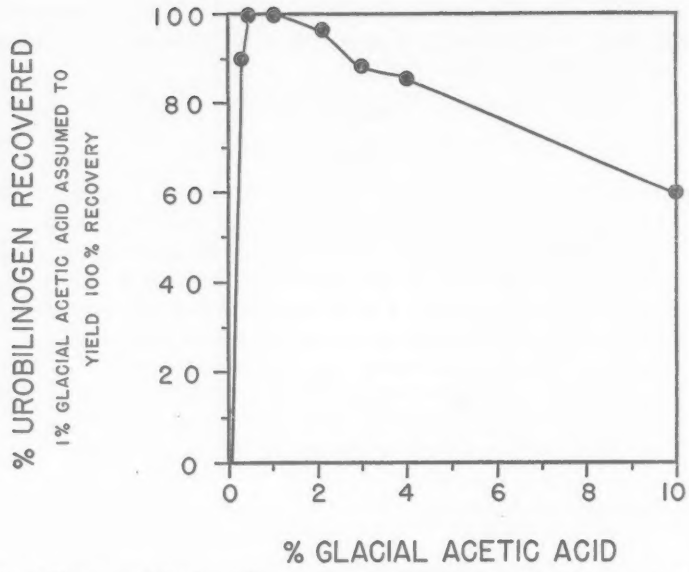

Fig. 2. Recovery of fecal urobilinogen from varying glacial acetic acid concentrations.

Table 1. Relation between Concentration of Acetic Acid and *p*H of Ferrous Hydroxide Filtrate

Glacial acetic acid concentration (%)	*p*H	
	5 ml. of filtrate	*10 ml. of filtrate*
	URINE	
0.5	4.4	4.9
1.0	4.0	4.3
2.0	3.7	3.9
3.0	3.5	3.8
4.0	3.3	3.6
	FECES	
0.1	6.1	11.6
0.5	4.0	4.4
1.0	3.7	3.9
2.0	3.3	3.6
3.0	3.2	3.4

acetic acid are shown in Table 1. A Beckman *p*H Meter Model G was used for all *p*H measurements.

Feces

Identical experiments were carried out on feces as described for urine, above. Glacial acetic acid concentrations ranged from 0.1 to 10%. From Fig. 2, it can be seen that the optimum concentration of glacial acetic acid is between 0.5% and approximately 1.5%. Here also similar results were obtained using 5 or 10 ml. of filtrate for extraction.

Determination of *p*H over the critical range of 0.1 to 3.0% glacial acetic acid are shown in Table 1.

The experiments were repeated on 5 separate samples of both urine and feces and yielded identical results in each instance.

DISCUSSION

Various references (2, 3) to the methods for extraction of urobilinogen from urine and feces are somewhat vague as to the specific degree of acidification optimum in the procedure. The importance of this aspect of the method has been a matter of some speculation in this laboratory. We were gratified, therefore, to note that optimum extractions were obtained uniformly when acidification of urine filtrates were held rigidly at the end concentrations of 2.0% glacial acetic acid, and the fecal filtrates at 1%. We have, therefore, instituted the following modifications of the standard Watson method referred to above.

Urine

Either 5 or 10 ml. of ferrous hydroxide filtrate is transferred to a separatory funnel. Water is added to bring the volume to 49 ml. One ml. of c.p. glacial acetic acid is added and the solution is mixed. Petroleum benzine is added and the analysis is continued as outlined by Watson and co-workers (1, 4).

Feces

The ferrous hydroxide filtrate is treated precisely as is urine except that water is added to bring the volume to 49.5 ml. and 0.5 ml. of C.P. glacial acetic acid is added.

The above findings have suggested that additional study of urobilinogen at various *p*H levels and its possible solubility in organic acids would be of value. Such a study will be undertaken shortly.

It is not surprising that the optimum acetic acid concentration is less for the fecal ferrous hydroxide filtrate than for the urine since the former is less alkaline than the urine.

The significance of the plateaus in Figs. 1 and 2 between acid concentrations of 3 and 4% is not known.

SUMMARY

A procedure has been discussed whereby the optimum extraction of urobilinogen from feces and urine can be accomplished by standardization of acidulation of the ferrous hydroxide filtrates in the Watson method.

REFERENCES

1. Schwartz, S., Sborov, V., and Watson, C. J., *Am. J. Clin. Path.* **14**, 598 (1944).
2. Watson, C. J., *Arch. Int. Med.* **47**, 698 (1931).
3. Watson, C. J., *Am. J. Clin. Path.* **6**, 458 (1936).
4. Watson, C. J., and Hawkinson, V., *Am. J. Clin. Path.* **17**, 108 (1947).

Cross-Contaminator Exclusion Test for Dry-Ashing Determination of Protein-Bound Iodine

Meyer Samson, Herman Brown, and Seymour Eichen

OXIDATION OF ORGANIC MATERIAL as a preliminary to determination of iodine, whether by wet or dry methods, entails loss of iodine if insufficient organic matter is present during the reaction (1, 2, 3). In blood serum, the organic content suffices to prevent this loss when the amount of iodine in the specimen is in the range found in physiological or pathological sera, 0–30 μg./100 ml., as has been repeatedly shown in recovery experiments (4, 5, 6, 7). When the iodine content of serum exceeds 500 μg./100 ml., as is sometimes the case after administration of iodinated radiopaque media, or other contact with iodinated medication, iodine may be volatilized in the ashing stage of the analysis, to be reabsorbed in other tubes. Such cross-contamination invalidates the entire series of tests, with loss of analyst's time and delay of reports.

To avoid delays caused by the presence of such cross-contaminators, a simple method of screening them out in advance was desirable. In connection with the quantitative dry-ashing procedure in use in this laboratory (7), the cross-contaminator exclusion test described below has proven satisfactory. It requires only 1 drop of serum, and adds no more than 30 minutes of operating time to a series of 20–40 PBI determinations.

REAGENTS AND APPARATUS

* 4N Sodium carbonate in dropper bottle.

Approximately 4N hydrochloric acid in dropper bottle.

* Approximately 0.15N arsenious acid in dropper bottle.

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* These items are identical with those used in quantitative PBI procedure (7).

* Approximately 0.065N ceric ammonium sulfate in dropper bottle.
* Muffle furnace, with control for 600° ± 25°.
Automatic electric Time Switch.[1]
Twin infrared heater with reflectors.[2]
Stainless steel test tube rack, 6 rows of 15 x 14 mm. holes.[3]
Pyrex test tubes with lip, 75 x 12 mm.

PROCEDURE

1. Place 1 drop (0.05 ml.) of serum and 2 drops of 4N Na_2CO_3 into a test tube. Shake rack. Dry completely under infrared heater.

The serum is dropped from the pipet used for the quantitative tests. Step 1 is set up simultaneously with the start of protein precipitation for the quantitative work. The Fisher Infra-rediator effects good drying in 2 hours if the lamps are adjusted about ½ in. from the tops of the tubes.

2. Put rack into muffle furnace preheated to 600°. Set automatic time switch to shut off at 2½ hours.

Step 2 is started at about the same time that the material in the quantitative tests goes into the drying oven.

3. To the cold ash, add 2 drops 4N HCl plus 1 drop arsenious acid solution. Shake. Allow to stand ten minutes. Add 1 drop ceric ammonium sulfate solution. Shake. Allow to stand 5 minutes. Read colors over a white background immediately at the end of 5 minutes, looking downward through tubes. Decolorized tubes indicate potential cross-contaminators.[4]

Step 3 is carried out the morning after Step 2. Reading of cross-contaminator exclusion tests is complete by the time the muffle is preheated to 600° for the quantitative determinations.

To guard against reagent deterioration, a blank containing Na_2CO_3 only, as well as a normal serum and one to which inorganic iodide equivalent to 400 μg. of iodine per 100 ml. has been added, are included in the qualitative series. The first two should be clear yellow at 5 minutes, the last completely decolorized.

EXPERIMENTAL DATA

When the need for a test to exclude cross-contaminating artefactual sera from the muffle furnace stage of dry ashing PBI determinations

[1] Electric Hotpack Company, Inc., Philadelphia: #6080B Limitor.
[2] Fisher Scientific Company, Pittsburgh: Heater, Infra-rediator, #11-504-5.
[3] Drummond Scientific Company, Philadelphia.
[4] Specimens decolorizing in 5 minutes are withdrawn from the quantitative ashing procedure. The dried proteinate, however, is not discarded. Instead, it is ashed separately and run through the quantitative procedure as usual, as a verification of the qualitative test.

became apparent, a search was made for a simple qualitative procedure. Feigl's (10) spot plate test for iodine appeared most promising, since its reagents were similar to those used in most quantitative procedures for serum iodine. It was accordingly adapted to serum ash. Pyrex test tubes were found to be the best ashing containers, as in quantitative work, since iodine losses occurred during ashing in shallow platewells or microcrucibles.

RESULTS

Typical results appear in Table 1. Decolorization of cerium within 5 minutes after its addition to the acidified and arsenized ash solution was regularly obtained at a level of 0.2 μg. I_2 per drop (0.05 ml.) of serum,

Table 1. Decolorization Times of Ceric Solutions

Iodine (μg./100 ml.)	*I_2 plus reagents unmuffled (min.)*	*I_2 plus reagents muffled (min.)*	*I_2 plus serum and reagents muffled (min.)*
100	Y 10	Y 10	Y 10
200	D 3	D 4	D 7
400	D 1	D 3	D 4
800	D 0.75	D 1.5	D 3

Y = yellow; D = decolorized.

equivalent to 400 μg. I_2/100 ml. This finding was not affected by the source of the added iodine, whether inorganic, as thyroxin, or as artefactual serum with an unknown iodine compound content. To avoid occasional cross-contamination during the qualitative muffling by sera containing very large amounts of iodine (>9000 μg./100 ml.), the proportion of carbonate to serum was increased to double that in the quantitative procedure. The effectiveness of this measure has led to consideration of the possibility of improvement of quantitative dry-ashing procedures in the same direction, a matter now being studied in our laboratory.

DISCUSSION

Practical methods for the determination of iodine in the fractional microgram amounts present in small serum samples depend on the catalytic acceleration of the reduction of ceric salts by arsenite in acid solutions, first described by Kolthoff and Sandell (8). While with the aid of instrumental analysis (9), this reaction is capable of differentiating increments of inorganic iodide in pure aqueous solution as small as 0.02 μg./100 ml., its sensitivity in qualitative work, in which the distinction

is between clearly visible yellow and colorlessness, is considerably lower. Feigl's (10) spot plate modification of the Kolthoff-Sandell reaction is read at 30 minutes, for which time an identification limit of 0.05 μg. KI per drop is given, equivalent to 75 μg. iodine per 100 ml. The sensitivity of the serum cross-contaminator exclusion test described above, for which a time limit of 5 minutes has been established for convenient inclusion in the quantitative procedure, and which contains much sodium chloride, is of the order of 400 μg. per 100 ml., or one twenty-thousandth that of increment differentiation under optimum conditions. It is nevertheless sufficient for the purpose. Concentrations of iodine below this level have not, in experience with the method in use in this laboratory, caused cross-contamination. If further analytical experience, or the advent of iodinated medications of cross-contaminating capacity at lower concentration, makes a more sensitive test desirable, one may be readily adapted from the experimental data set forth.

Not all artefactual sera have the same cross-contaminating potential. Some sera having iodine content upward of 5000 μg./100 ml. did not cross-contaminate known control sera included in the series. On the other hand, some artefactual sera with iodine content one tenth the above level have been implicated. It is evident that volatilization of iodine depends to a large extent on the nature of the compound present. Further study to evaluate the analytical characteristics of the various iodinated medications apt to be found in serum may prove valuable.

SUMMARY

A simple qualitative test for iodine in blood serum has been described. It is designed to detect sera which are potential cross-contaminators in dry-ashing PBI procedures. One drop of serum is used. Added operating time in a PBI series of 20–40 determinations is not over 30 minutes.

REFERENCES

1. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.* **163**, 313 (1946).
2. Barker, S. B., *J. Biol. Chem.*, **173**, 715 (1948).
3. Association of Official Agricultural Chemists, Washington, D. C., *Methods of Analysis*, "Iodine in Drugs-official", p. 597 (1950).
4. Decker, J. W., and Hayden, H. J. *Anal. Chem.* **23**, 798 (1951).
5. Barker, S. B., Humphrey, M. J., and Soley, M. H., *J. Clin. Invest.* **30**, 55 (1951).
6. Man, E. B., Kydd, D. M., and Peters, J. B., *J. Clin. Invest.* **30**, 531 (1951).
7. Brown, H., Reingold, A. M., and Samson, M., *J. Clin. Endocrinol. and Metabolism* **13**, 444 (1953).
8. Kolthoff, I. M., and Sandell, E. B., *J. Am. Chem. Soc.* **56**, 1426 (1934).
9. Chaney, A. L., *Anal. Chem.* **22**, 939 (1950).
10. Feigl, F., *Spot Tests*, Houston, Texas, Elsevier Press, 1954, Vol. I, p. 250.

the Clinical Chemist

EDITORIAL

At the time this is being written, a lawsuit is in process in Des Moines, Iowa, to determine whether hospitals are engaging in the practice of medicine when they own and operate laboratories and hire specialists to work in these laboratories. This suit was initiated by the Iowa Hospital Association and 34 member hospitals, and they have requested the court, with District Judge C. Edwin Moore presiding, to overrule the Iowa Attorney General's opinion, that hospitals owning and operating laboratories are practicing medicine illegally. Defendants in this action are the Attorney General's Office, the Iowa State Board of Medical Examiners, and the Iowa Association of Pathologists.

The clinical chemist is not a principal in this controversy, yet he obviously will be very directly affected in any decision as to what constitutes the practice of medicine as it relates to laboratory procedures. The American Association of Clinical Chemists has contended from the beginning that clinical chemistry is not the practice of medicine and the chemist is not engaged in the practice of medicine when he performs chemical determinations requested by a physician, or when he discusses these determinations with a physician who may seek information in the interest of his patient. These concepts are clearly stated in the Resolution on Legislation and in the Code of Ethics of the American Association of Clinical Chemists.

The Association does not of course take any official position in this Iowa controversy. The points of conflict are far-reaching and extend much beyond the

chemistry laboratory. But the Association is prepared, however, to defend the rights of the clinical chemist at all times to practice his specialty at the highest professional and scientific level. This is in the public interest.

MAX M. FRIEDMAN
National Secretary

Following are accounts of the testimony that appeared in the Des Moines, Iowa, *Register* and *Tribune*, May 24 and 25, 1955. (Reprinted by permission of the publishers.)

Says Chemists Are Underpaid

By Walter Shotwell.

Clinical chemists associated with hospitals or universities generally are "miserably underpaid and overworked," Dr. John G. Reinhold, Glen Mills, Penn., testified Monday in Polk county district court.

But, he added, money is a "secondary consideration" because "a good scientist usually is a dedicated man."

Second Witness.

Senior consultant in biochemistry to the United States army's surgeon, Dr. Reinhold was the second witness called by the Iowa Hospital Association in a trial to determine whether Iowa hospitals may legally operate laboratories staffed with hired specialists.

Dr. Reinhold said in response to questions that never in his experience has a hospital administration interfered with his professional judgment.

He testified that he prefers employment with a hospital because he feels that employment by an individual pathologist would limit the "free relationship between physician and scientist" and the scope of scientific research.

He termed it "highly significant" that few laboratory techniques have been developed by medical doctors, explaining that "they haven't had the interest."

Still, Dr. Reinhold testified, "We (the U. S.) lead the world in clinical chemistry."

Under cross-examination, Dr. Reinhold testified that he regards his responsibility as providing information to the physician, who then makes appropriate decisions.

He said no relationship exists directly between the chemist and the patient and that the chemist receives no money from the patient. However, he said the chemist will occasionally view the patient's medical record during consultation with the physician.

Friedman Testifies.

Also testifying Tuesday afternoon was Dr. Max M. Friedman, Rego Park, N. Y., chemist of Lebanon Hospital in New York, N. Y.

Dr. Friedman testified as to contributions made by clinical chemists to medical advancements but said chemists "are not competent" to practice medicine as such.

He testified that if individual clinical chemists were required to work only for individual pathologists, it "would hamper the progress of biochemistry, discourage well-trained chemists" from entering the medical field and would force chemists to work with poor facilities.

Chemists also would have less time for research, Dr. Friedman testified. He said he "disagrees violently" with one leading chemist who predicted in a nationally-circulated journal that in the future chemists may actually treat patients.

Dr. Friedman also denied under cross-examination that "formidable warfare" exists between chemists and pathologists. However, he admitted that the chemists have been seeking legislation to grant them "recognition."

AACC ELECTIONS

President

Otto Schales, of the Alton Ochsner Clinic in New Orleans, La., was elected President of the American Association of Clinical Chemists to succeed Lt. Col. Monroe E. Freeman, whose term of office expired on June 30, 1955. Dr. Schales has served the Association in many capacities and was Vice-President for the past year. He is well known to his colleagues for his scientific and professional attainments. His term of office is to extend until June 30, 1956.

Officers

The other officers elected for the same period were Robert M. Hill, University of Colorado Medical School, Vice-President; Max M. Friedman, Lebanon Hospital, New York City, National Secretary; and Louis B. Dotti, St. Luke's Hospital, New York City, National Treasurer.

Executive Committee

The new Executive Committee will consist of the above officers in addition to the following members: Monroe E. Freeman, Medical Service Corps, Washington, D. C.; Joseph H. Gast, Baylor University School of Medicine; William H. Goldwater, U. S. Naval Radiological Defense Laboratory, San Francisco; Icie M. Hoobler, Children's Fund of Michigan, Detroit; and Samuel Natelson, Rockford Memorial Hospital, Rockford, Ill.

Nominating Committee

The new Nominating Committee elected by the membership will consist of John G. Reinhold, chairman; Miriam Reiner, District of Columbia General Hospital; Harry Sobotka, Mount Sinai Hospital, New York; Joseph I. Routh, University Hospitals, Iowa City; Arnold G. Ware, Los

Angeles County Hospital; Albert E. Sobel, Jewish Hospital of Brooklyn; and Warren M. Sperry, N. Y. State Psychiatric Institute, New York.

It will be the function of this Nominating Committee to propose a slate of officers and members for the 1956–57 Executive Committee.

The ballots were counted and the election certified by Julius J. Carr, Albert Hanok, and Andre C. Kibrick.

1955 ANNUAL MEETING OF THE AACC

The Annual Meeting of the American Association of Clinical Chemists will be held once again with the Fall National Meeting of The American Chemical Society in Minneapolis, Minn., sometime during the week of September 11–16, 1955. The exact dates are not yet available and since the next issue of CLINICAL CHEMISTRY will not appear until after the meeting it will be necessary for our members to obtain the meeting program from *Chemical and Engineering News*. The final program will appear in the August 8 issue of *Chemical and Engineering News*, in which all the Association events will be scheduled with the exception of the membership meeting. In the past this meeting was held immediately following the symposium with the Division of Biological Chemistry and preceding the Annual Dinner.

The social highlight of the year has been the annual dinner at which the Ernst Bischoff Award is presented. The success of these dinners can be best attested to by the fact that for the one held last year in New York it was necessary to set up tables in the alcoves to accommodate the overflow from the dining room.

SPANISH TRANSLATION OF *STANDARD METHODS*

Arrangements have been made with a publishing firm in Madrid for the translation of *Standard Methods in Clinical Chemistry* into the Spanish language. The Association will receive royalties for the translated edition as for the English edition.

ENZYME SYMPOSIUM

A three-day International Symposium on "Enzymes: Units of Biological Structure and Function," sponsored by the Henry Ford Hospital and The Edsel B. Ford Institute for Medical Research, will be held in the auditorium of Henry Ford Hospital, Detroit, Mich., November 1, 2, and 3, 1955.

Interrelationships between enzymology and other fields—notably genetics, physiology, biochemistry, and pharmacology—will constitute the general theme of the symposium. The specific topics for the six sessions will be: Origin of Enzymes, Status of the Gene-Enzyme Relationship, Enzymes and Cell Structure, Enzymatic Basis of Some Physiological Functions, Cellular Energy Sources, and Regulation of Enzyme Activity. More than thirty internationally known scientists have accepted invitations to participate.

A copy of the Preliminary Announcement may be obtained by writing to Dr. Clarence E. Rupe, Henry Ford Hospital, Detroit 2, Mich.

Invitations will be sent to as many as can be accommodated.

AAAS ANNUAL MEETING

The American Association of Clinical Chemists will participate in the 122nd meeting of the American Association for the Advancement of Science to be held in Atlanta, Ga., December 26–31, 1955. The preliminary program has already been published in *Science*, May 12, 1955, page 751. The Association, with Albert E. Sobel as program chairman, has arranged a symposium on "Recent Concepts in Clinical Chemistry" and a dinner on December 26. There will also be two sessions for contributed papers in clinical chemistry on December 27.

This is the first occasion for the Association to collaborate with the AAAS in their national meetings. It is to be hoped that our members will be well represented at these meetings and that this collaboration will become an annual event. The Association has been an affiliated society of the AAAS for several years.

AMERICAN BOARD OF CLINICAL CHEMISTRY

The American Board of Clinical Chemistry, Inc., held its annual meeting at the Henry Ford Hospital, Detroit, Mich., on May 20–21, 1955. Three clinical chemists who successfully passed examinations given by the board last October were certified: Sol I. Bulkin, Lawrence C. Kier, and Otto E. Loebstein. The total number of certified clinical chemists is now 241.

A complete *Directory of Certified Clinical Chemists* may be obtained by writing to the Secretary-Treasurer, Dr. O. H. Gaebler, Henry Ford Hospital, Detroit, Mich., and will be sent without charge to hospital departments or other laboratories which engage in clinical chemistry.

Clinical chemists interested in being certified should write for the *Instructions to Applicants for Certification*. In order to be considered for admission to the next regional examination, which will probably be held during October, 1955, candidates should file their applications immediately.

The Board elected the following officers for the coming year: Marschelle H. Power, *President*; Clarence W. Muehlberger, *Vice President*; and Oliver H. Gaebler, Secretary-Treasurer. The other members of the Board are: Joseph W. E. Harrisson, Arnold E. Osterberg, William A. Wolff, Warren M. Sperry, Harry Sobotka, Robert M. Hill, and Albert L. Chaney.

REPORTS FROM THE SECTIONS

Southern California

Clyde A. Dubbs

The third symposium of the season was held on February 1 at the Los Angeles County Hospital. Robert Stragnell and Gerard Lanchantin, both of the University of Southern California Medical School, discussed "Recent Advances in Blood Coagulation and Their Clinical Significance."

ABSTRACTS

Editor: Ellenmae Viergiver. *Contributors:* Joseph A. Annino, Gladys J. Downey, Clyde A. Dubbs, Alex Kaplan, Margaret M. Kaser, Miriam Reiner, Herbert Thompson

Effect of glucagon on peripheral utilization of glucose in man. T. B. van Itallie, M. C. Morgan, and L. B. Dotti (*St. Luke's Hospital, New York, N. Y.*).

Peripheral glucose uptake in normal human subjects during and after glucagon-induced hyperglycemia was compared with peripheral uptakes during and after similar hyperglycemias produced by intravenous infusion of glucose, and by ingestion of glucose following subcutaneous injection of epinephrine. Comparison of CV differences following glucagon administration with those following the infusion of glucose showed that peripheral utilization of glucose was not inhibited by glucagon and may have been enhanced. In contrast, epinephrine injection was followed by apparent inhibition. It is suggested that the description of glucagon as an "insulin antagonist" is misleading.—*J. Clin. Endocrinol. and Metabolism* **15,** 28, 1955. (M. R.)

Effect of long-term administration of desiccated thyroid on serum lipoprotein and cholesterol levels. B. Strisower, J. W. Gofman, E. Galioni, J. H. Rubinger, G. W. O'Brien, and A. Simon (*University of California, Berkeley, Calif.*).

In a series of 50 schizophrenic patients receiving a daily dosage of 3 gr. of desiccated thyroid, the concentrations of the mean Standard S_f0-12 and mean Standard S_f12-20 serum lipoproteins were significantly reduced within a period of 3 weeks, but by 24 weeks the Standard S_f0-12 concentration had returned to the prethyroid level. The magnitude of the drop in the Standard S_f0-12 concentration was directly related to the prethyroid value.—*J. Clin. Endocrinol. and Metabolism* **15,** 73, 1955. (M. R.)

The determination of sugar in blood and spinal fluid with anthrone reagent. J. H. Roe (*George Washington University, Washington, D. C.*).

Glucose in blood or spinal fluid is determined by first deproteinizing with trichloroacetic acid, tungstic acid, or barium hydroxide-zinc sulfate. Anthrone reagent is then added to the filtrate, the solution is heated in a boiling water bath, and the density of the resultant color is measured at 620 mμ. An anthrone reagent containing thiourea is described which is stable for two weeks. Comparisons of results with the standard copper reduction methods are in good agreement.—*J. Biol. Chem.* **212,** 335, 1955. (J. A.)

Progressive changes in renal phosphate and calcium excretion in rats following parathyroidectomy or parathyroid administration. R. V. Talmage and F. W. Kraintz (*Rice Institute, Houston, Texas*).

The urinary excretion of Ca^{45} or P^{32} was followed for 27 hours after their administration to adult rats 1 hour after parathyroidectomy or after the first injection of parathyroid extract, and serum calcium and phosphorus were determined in animals sacrificed at intervals during the experimental period. Parathyroidectomy caused an increase in the urinary excretion of calcium for at least 12 hours and an immediate drop in urinary phosphorus, but at 24–27 hours the rates of excretion of both ions had returned to normal. Throughout the experimental period the serum calcium fell and serum phosphorus rose. The results following parathyroid extract were in the opposite directions, but the rates were comparable. It is concluded that the control of the renal thresholds for both calcium and phosphate by parathyroid hormone is the cause of the altered serum levels observed, and that any changes in excretion occurring after 24 hours are due to extrarenal effects of parathyroid administration or deprivation.—*Proc. Soc. Exp. Biol. Med.* **87**, 263, 1954. (M. K.)

Simultaneous measurement of the iodide-concentrating and protein-binding capacities of the normal and hyperfunctioning human thyroid gland. S. H. Ingbar (*Walter Reed Army Medical Center, Washington, D. C.*).

It is possible in man to measure simultaneously the ability of the thyroid gland to concentrate inorganic iodide and to bind thyroidal iodide to protein as shown in a study of 29 normal individuals and 20 patients with untreated Graves' disease. The increased rate of hormone synthesis in the latter group was usually the result of increases in both the iodide-concentrating and protein-binding capacities of the thyroid gland. The method also makes possible the measurement of the rate of transfer of plasma iodide to hormonal iodine which can be applied to patients receiving large doses of antithyroid drugs.—*J. Clin. Endocrinol. and Metabolism* **15**, 238, 1955. (M. R.)

A rapid, simple method for the determination of reduced, dehydro-, and total ascorbic acid in biological material. R. R. Schaffert and J. R. Kingsley (*University of California, Los Angeles, Calif.*).

Total ascorbic acid is determined as follows: a protein-free filtrate is prepared using 6% trichloroacetic acid. The filtrate is treated with Norit (amorphous carbon) and filtered. To the filtrate is added 10% thiourea and 2,4-dinitrophenylhydrazine. The mixture is then heated in a water bath. After cooling, 85% H_2SO_4 is added and the resultant color density is measured at 515 mμ. When the procedure is carried out omitting the Norit, dehydroascorbic acid is measured. The L-ascorbic acid is the difference between the total and the dehydroascorbic acid.—*J. Biol. Chem.* **212**, 59, 1955. (J. A.)

Effects of intravenous hydrocortisone on electrolytes of serum and urine in man. R. P. Knight, D. S. Kornfeld, G. H. Glaser, and P. K. Bondy (*Yale University School of Medicine, New Haven, Conn.*).

When hydrocortisone was given in large amounts (50 or 100 mg.) intravenously over a period of 4 hours to 5 subjects, the blood eosinophil count fell, but urine and serum sodium and chloride levels failed to exhibit a consistent pattern of response. It was also possible to demonstrate a heretofore undescribed early increase in the serum potassium level concomitant with the expected rise in urine potassium. These increases in the serum potassium ranged from 0.6 to 1.1 mEq. in 4 of the 5 subjects over the 4 hours. This suggests that one of the major early effects of hydrocortisone on electrolyte metabolism is on the distribution of cations between intracellular and extracellular fluid since the increased urinary potassium excretion induced was insufficient to prevent a rise of the serum potassium.—*J. Clin. Endocrinol. and Metab.* **15,** 176, 1955. (M. R.)

Determination of estrogens in stored urines of pregnancy. R. M. Anker (*University of Colorado Medical School, Denver, Colo.*).

The Allen modification of the Kober colorimetric assay was investigated and improved. The uncertainty is estimated to be at most 300 μg. per 24-hour urine specimen, depending upon the colored impurities present in the urine. The specimens may be stored for 2 years at room temperature under a layer of *n*-butanol; the recovery is much better than when aqueous mineral acid is used as the means of clearing the conjugates.—*J. Clin. Endocrinol. and Metabolism* **15,** 210, 1955. (M. R.)

Quantitative determination of urine sodium by means of ion-exchange resins. J. S. Vanatta and C. C. Cox (*University of Texas, Dallas, Texas*).

A modification of a serum sodium technic previously published (*J. Biol. Chem.* **210,** 719, 1954) is described. Urine is passed through IR-112 which removes sodium. The sodium is eluted with $BaCl_2$ solution and the barium is precipitated as $BaSO_4$. The supernatant is then passed through IRA-400 to convert the NaCl to NaOH. Subsequently the NaOH is titrated with standard HCl using bromthymol blue as an indicator. Data are presented showing good agreement with the Butler and Tuthill method.—*J. Biol. Chem.* **212,** 599, 1955. (J. A.)

Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. P. R. N. Kind and E. J. King (*Postgraduate Medical School, London, England*).

The method of Grifols-Lucas (*Brit. Med. J.* **2,** 295, 1951) has been modified by the present authors to yield results comparable to those obtained by the King-Armstrong procedure. The amount of phenol liberated by enzymatic hydrolysis is determined by reaction with 4-amino-antipyrine (AAP). A red-colored quinone

is produced which can be measured colorimetrically. Proteins do not react with AAP and therefore need not be removed.—*J. Clin. Pathol.* **7,** 322, 1954.

(E. V.)

The endogenous creatinine clearance in normal subjects. M. H. Roscoe (*Manchester University, Manchester, England*).

The data presented in this paper show that the excretion of endogenous creatinine is very constant in spite of considerable variation in urine flow, decreasing only slightly at low urine volumes. The creatinine clearance was lower than the expected filtration rate as determined by the clearance of inulin. It is suggested that this discrepancy is probably due to overestimation of the plasma creatinine. If, as has been found by Roscoe (*J. Clin. Pathol.* **6,** 201, 1953), 20 per cent of the estimated chromogen is not creatinine, the differences in the clearances can be accounted for.—*J. Clin. Path.* **7,** 327, 1954. (E. V.)

Permanent color standards for determination of phosphate by molybdenum blue method. E. P. Parry and A. L. McClelland (*University of Connecticut, Storrs, Conn.*).

Stable color standards, having hue and absorption spectrum (400 to 700 mμ) similar to molybdenum blue, are prepared. A standard containing 4.98 mg. of copper sulphate and 0.920 mg. of bromophenol blue in 10 ml. of acetate buffer (*p*H 4.63) showed no absorption spectrum change when exposed for 24 days in the direct morning sunlight.—*Anal. Chem.* **27,** 140, 1955. (C. D.)

Silica gel microcolumn for chromatographic resolution of cortical steroids. M. L. Sweat (*University of Utah College of Medicine, Salt Lake City, Utah*).

As little as 0.25 μg. of a steroid can be chromatographically determined using a 40 $\times$ 5 mm. O.D. microcolumn containing 0.1 Gm. of silica gel. The column is suspended in a water-jacketed condenser having a stopcock outlet. Corticosterone and 17-hydroxycorticosterone can be determined in 2 ml. of plasma. A synthetic mixture was resolved, by elution with 100 ml. of alcohol-chloroform, into desoxycorticosterone, 17-hydroxy-11-desoxycorticosterone, corticosterone, cortisone, 17-hydroxycorticosterone, and 4-pregnene-11β, 17α, 20β, 21-tetrol-3-one in that order of elution. While some overlapping occurred, the relative specificities of the fluorescent and phenylhydrazine analytical methods permit satisfactory individual determinations in most cases. Applications to human, dog, and rat blood are described elsewhere.—*Anal. Chem.* **26,** 1964, 1954.

(C. D.)

Potassium and reducing substances in proximal tubule fluid of the rat kidney. H. Wirz and P. A. Bott (*Women's Medical College of Pennsylvania, Philadelphia, Pa.*).

The kidneys of rats were exposed, the middle third of the proximal tubule of a single nephron was isolated and punctured as described by Walker and Oliver (*Am. J. Physiol.* **134,** 562, 1941), and a ureter was cannulated. Potassium in plasma, tubule fluid, and urine was determined by means of an internal standard flame photometer and reducing substances by an ultramicro adaptation of the Sumner method as described by Walker and Reisinger (*J. Biol. Chem.* **101,** 223, 1933).

Lower concentrations of potassium in tubule fluid than in plasma, together with reabsorption of water in the proximal tubule of the rat kidney, demonstrate that considerable reabsorption of potassium takes place. This is also the site of active glucose reabsorption.—*Proc. Soc. Exp. Biol. Med.* **87,** 405, 1954.

(M. K.)

Effects of hormone administration on serum protein patterns. C. H. Ligeti and K. Irvine (*School of Medicine, University of Virginia, Charlottesville, Va.*).

Previous studies have revealed the occurrence of lowered serum albumin levels and increases in the α_1- and α_2-globulins after surgery. In these experiments large doses of cortisone increased the total serum protein concentration, but no other hormone tested had a significant effect. Serum albumin was decreased by pituitary adrenocorticotropic hormone and increased by testosterone, hydrocortone, and cortisone. Insulin increased α_1-globulin and estrogen increased the α_2-globulin. Serum γ-globulin was depressed by hydrocortone, but not by cortisone. The other pituitary and adrenal hormones, progesterone, and thyroid had no effect on serum protein patterns. *Proc. Soc. Exp. Biol. Med.* **87,** 324, 1954.

(M. K.)

Occurrence of glucose in combination with acetoacetate in normal urine. M. C. Nath and V. K. Sahu (*University Department of Biochemistry, Nagpur, India*).

Previously a metabolic relationship between glucose and acetoacetate in rabbits had been demonstrated by hypoglycemia followed by hyperglycemia after repeated injections of acetoacetate, the production of hyperglycemia by either glucose or acetoacetate but not by the two together, and the protection afforded by glucose and acetoacetate together or their condensation product (2-tetrahydroxy butyl, 5-methyl, 4-carbethoxy furan) against alloxan diabetes.

In the present experiments, urine from rats, rabbits, and normal and diabetic human subjects subjected to hydrolysis with HCl reduced Nylander's reagent and yielded glucosazone but did not do so before hydrolysis. Anthrone gave a marked color and tests for acetone were positive after hydrolysis. Unhydrolyzed urines gave a red-orange color with $FeCl_3$, as did the glucose-acetoacetate condensation product. Several urines from patients with glycosuria and acetonuria showed no increase in glucose or acetone with hydrolysis. The data indicate that

glucose and acetoacetate occur in normal urine in a combined form, which is not present in diabetes.—*Proc. Soc. Exp. Biol. Med.* **87**, 287, 1954. (M. K.)

Conjugates of adrenal corticoids in human plasma. A. M. Bongiovanni, W. R. Eberlein, M. M. Grumbach, J. J. VanWyk, and G. Clayton (*Johns Hopkins School of Medicine, Baltimore, Md.*).

Concentrated chloroform extracts of plasma chromatographed on Florosil and treated with phenylhydrazine according to Nelson and Samuels (*J. Clin. Endocrinol. & Metabolism* **12**, 519, 1952) constituted the "free" corticoids. The remaining plasma was acidified to *p*H 5.5 with 40% H_2SO_4 and then to *p*H 4.5 with acetate buffer. Beta-glucuronidase (1000 units/ml. plasma) was added and the plasma incubated at 37° for 24 hrs. Dialysis was carried out at room temperature for 48 hr. against 12 volumes of 10% methanol in water, and the dialysate was extracted with chloroform. The extracts were treated as for the "free" fraction.

Normal values for free corticoids ranged from 2 to 10 μg./100 ml. and comparable values were found for the conjugated corticoids in 8 patients without adrenal disease. Both were increased in two subjects after acute injuries; the free fraction was elevated in a child with adrenocortical carcinoma with metastases; and the results in 2 patients with Cushing's disease were variable but the proportion of conjugated steroids was elevated. ACTH and Compound E acetate caused increases in both free and conjugated fractions, with the conjugated portion exceeding the free after about two hours. Disposition of excessive hormone involves conjugation, which was impaired in cirrhosis of the liver. Evidence is presented to support the belief that the material measured in the conjugated fraction consists of corticoids.—*Proc. Soc. Exp. Biol. Med.* **87**, 282, 1954.

(M. K.)

Comparative value of the basal metabolic rate, chemical protein-bound iodine, and radioactive iodine excretion or uptake in the diagnosis of borderline hyperthyroidism when used individually or in combination. L. Zieve, B. Skanse and A. L. Schultz (*Veterans Administration Hospital and University of Minnesota, Minneapolis, Minn.*).

Considered individually, radioactive iodine uptake, or excretion, in 24 hours is the most valuable index for distinguishing between borderline euthyroid and borderline hyperthyroid patients. Protein-bound iodine is about 80 per cent as effective, and the basal metabolic rate about 20 per cent as effective. A weighted combination of protein-bound iodine and radioactive iodine uptake is better, although not greatly so, than either one alone. Both are needed for maximum diagnostic effectiveness.—*J. Lab. Clin. Med.* **45**, 281, 1955. (G. D.)

Correction for color differences between standards and urine extracts and their ketonic fractions in the Callow-Zimmerman reaction. H. P.

Schedl, W. B. Bean, B. M. Stevenson, and E. R. Schumacher (*State University of Iowa, Iowa City, Iowa*).

Following analysis of urine extracts for 17-ketosteroids, aliquots of the original ethanol solution were dried in vacuo. After Girard separation (Pincus and Pearlman, *Endocrinology* **29,** 413, 1941), both ketonic and nonketonic fractions were analyzed, all Zimmerman reactions being performed by the method of Callow (Callow, Callow, and Emmons, *Biochem. J.* **32,** 2, 1312, 1938). Since the Zimmerman reaction absorption spectra of ketonic substances in urine extracts are not the same as that of the standard, dehydroisoandrosterone acetate, and since destruction of chromogens occurs during the Girard procedure, uncorrected analyses on ketonic fractions may be misleading. Using measurements made at three wave lengths—425, 510, and 595 mμ—the authors propose that the 17-ketosteroid content of the sample be corrected by comparing the absorption peak of the unknown with that of the standard.—*J. Lab. Clin. Med.* **45,** 191, 1955. (G. D.)

Turbidimetric method of fibrinogen assay: Results with the Coleman Junior spectrophotometer. A. H. Fowell (*Cutler Laboratories, Berkeley, Calif.*).

The recent recognition of afibrinogenemia of pregnancy has emphasized the need for a rapid method of fibrinogen assay in blood. The method of Parfentjev, Johnson, and Clifton [*Arch. Biochem.* **46,** 470 (1953)] has been adapted for use with the Coleman Junior spectrophotometer, Model 6A. Five milliliters of blood are collected in a test tube containing 0.5 ml. of 4% sodium citrate dihydrate. Plasma is separated by centrifugation at 2500 rpm for 10 minutes. The blank is 1 ml. of plasma plus 9 ml. of normal saline; the sample is 1 ml. of plasma plus 9 ml. of Parfentjev reagent (133.33 Gm. of ammonium sulfate, 10.0 Gm. of sodium chloride, and 0.25 Gm. of Merthiolate in 1 liter of aqueous solution; *p*H is adjusted to 7.00 with 10M NaOH). The spectrophotometer is set to read 100% transmittance at 510 mμ with the blank. The optical density of the sample is measured at exactly 3 minutes after the addition of the reagent. If coagulation has occurred, the cuvette is given a vigorous shake just before reading. Fibrinogen concentration of the sample in grams per 100 ml. equals $\frac{\text{O.D.} + 0.019}{0.509}$.

The lower levels of fibrinogen, 0.05 to 0.150 Gm./100 ml., where fibrinogen replacement therapy is required, will be clearly indicated by this method.—*Am. J. Clin. Pathol.* **25,** 340 (1955). (H. T.)

Determination of quinine in urine in "tubeless method" of gastric analysis. R. D. Lewis and A. G. Foord (*Huntington Memorial Hospital, Pasadena, Calif.*).

Modifications of the "tubeless method" of gastric analysis of Segal, Miller, and Morton [(*Proc. Soc. Exp. Biol. Med.* **74,** 218 (1950)] are described. The authors

substituted chloroform for ether in the urine extraction because in their experience recoveries were poor with the gentle ether extraction. Ten milliliters samples of urine are transferred to 16 × 100 mm. tubes and 0.5 ml. of 0.1N NaOH is added, followed by 5 ml. of chloroform. The tubes are covered with the thumb protected by a clean rubber cot and vigorously shaken for 10 seconds. They are then centrifuged and the urine phase is removed by aspiration. The chloroform is washed with 5 ml. H_2O and recentrifuged. The water phase is removed. Ten milliliters of 0.1N H_2SO_4 is added to the chloroform extract and the tubes are again shaken. The sulfuric acid phase (upper layer) is removed and the fluorescence of the specimen is compared with standards in ultraviolet light. Recoveries with ether were found to be 38–60 per cent; when chloroform was used recoveries were 76–93 per cent.—*Am. J. Clin. Pathol.* **25,** 199 (1955).

(H. T.)

Ultramicro procedures in clinical chemistry. W. T. Caraway and H. Fanger (*Rhode Island Hospital, Providence, R. I.*)

This is a report of the authors' study of procedures for all the common clinical chemistry determinations on 0.01 ml. samples of serum. Nearly all the procedures described can be adapted to larger samples by multiplying all quantities by a factor of 10 or 100. Details concerning equipment and capillary blood collection are given and procedures are described in detail for the ultramicro determination of glucose, urea nitrogen, creatinine, total protein, albumin, sodium, potassium, calcium, chloride, carbon dioxide combining power, inorganic phosphorus, icterus index, bilirubin, cholesterol, thymol turbidity, cephalin flocculation, alkaline phosphatase, and amylase. Only a small working area is required for these ultramicroprocedures but this should be used exclusively for this work. The procedures are somewhat more time-consuming than are the standard methods and are less adaptable to a large volume of work. Data shown demonstrate that the precision of the ultramicromethods is in conformity with that of standard accepted macro procedures.—*Am. J. Clin. Pathol.* **25,** 317 (1955).

(H. T.)

Identification of urinary sugar. F. W. Fales (*Emory University, Atlanta, Ga.*)

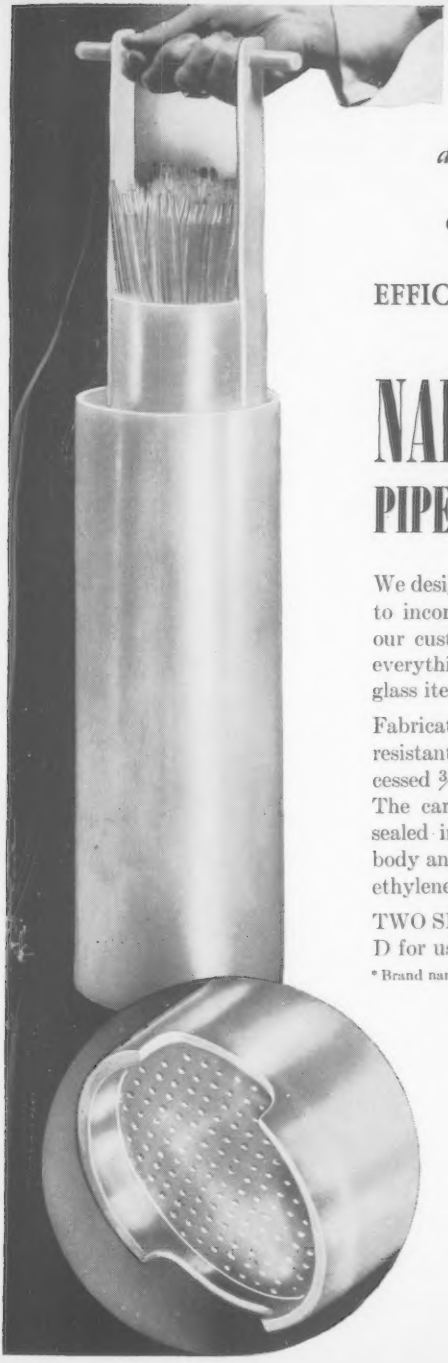
The presence of reducing sugar in urine is determined by Benedict's qualitative reagent. If the concentration is low, the urine is concentrated to 1% sugar by evaporation. A fermentation test is made to establish the presence or absence of fermentable sugar. If fermentation is present, the sugar may be glucose or fructose; if absent, either galactose, lactose, or pentose is present. Final identification of the unknown sugar is obtained by paper chromatography, using known sugars as controls.—*Am. J. Clin. Pathol.* **25,** 336 (1955). (H. T.)

Counter-current distribution procedure for the quantitative-qualitative analysis of small amounts of urinary corticosteroids. N. B. Talbot, S.

Ulick, A. Koupreianow, and A. Zygmuntowicz (*Harvard Medical School, Boston, Mass.*)

A 24-hour urine specimen (adjusted to *p*H 6) is extracted 4 times with 1/7 volume of chloroform. The chloroform extract is separated by centrifugation and washed 3 times with 1/10 volume of 0.1N NaOH and 3 times with 1/10 volume of water. The residue from this extract is dissolved in the contents of the zero tube of the distribution apparatus, which is filled with equal volumes of pure upper and lower layer solvents (iso-octane and secondary butanol). The distribution is performed according to Craig; the apparatus is gently inverted for 45 seconds at each transfer and checked for the separation of the two layers by direct observation. After 1 hour the material in each tube is transferred to one of a series of bottles. The contents are dried in vacuo and the residue is dissolved in 5 ml. of ethanol. The optical density is determined at 302 mμ (Value A). A 3 ml. aliquot is then transferred to a 15 ml. glass-stoppered test tube and 0.5 ml. of thiocarbazide reagent is added. After 90 minutes at room temperature the optical density is determined at 302 mμ. This value is multiplied by a factor of 1.17 to correct for the dilution caused by the addition of reagent (Value B). The increase in optical density at 302 mμ (Value C) attributable to thiosemicarbazones is found by subtracting Value A from Value B.

Clinical studies indicate that the excretion pattern per square meter per day of healthy persons is quite steady. Gross qualitative as well as quantitative changes in the urinary corticosteroids have been observed in a small series of patients suffering either severe stress or definite adrenocortical disease.—*J. Clin. Endocrinol. and Metabolism* **15,** 301 (1955). (M. R.)



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The Tablet That Makes

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As Easy as A-B-C

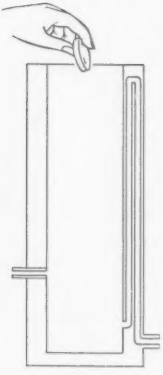
A DROP A TERG-A-TAB IN WASHER

B PLACE PIPETTES IN WASHER

C TURN ON HOT WATER

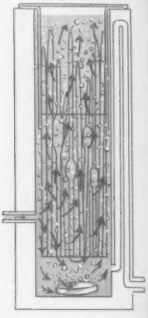
Let Terg-a-Tab Do the Rest!

TERG-A-TABS are a powerful, fast-acting detergent in tablet form. By slowly dissolving the tablet through an effervescing action, just the right amount of detergent is allowed to mix with the water, and the action of the washer alone is enough to make your pipettes sparkling clean. No other chemicals or cleansers are necessary. No film is left on your pipettes! One tablet will clean a full washer of pipettes!



EASY . . .

Simply drop a TERG-A-TAB into the pipette washer and place the rack containing the pipettes over the tablet. Then turn on the warm water (160°F). In warm water TERG-A-TAB slowly dissolves, making a detergent solution.

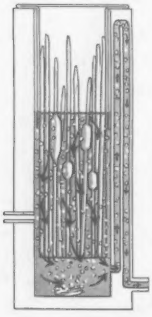


ECONOMICAL . . .

No measuring is necessary, as the right amount of detergent automatically dissolves in the water. The cleaning solution enters the pipettes from the bottom, going in and about them to clean all the surfaces.

EFFECTIVE . . .

As the washer automatically empties, detergent solution passes down, through and around the pipettes, carrying out loosened dirt. This action continues until TERG-A-TAB has completely dissolved. The dissolving time is approximately 8 to 10 minutes.



EFFICIENT . . .

After TERG-A-TAB has completely dissolved, fresh water enters and leaves the pipette washer, rinsing the pipettes and leaving them sparkling clean!

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